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# cDNAs of Cell Adhesion Molecules of Different Specificity Induce Changes in Cell Shape and Border Formation in Cultured S180 Cells

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*Abstract.* The liver cell adhesion molecule (L-CAM) and N-cadherin or adherens junction–specific CAM (A-CAM) are structurally related cell surface glycoproteins that mediate calcium-dependent adhesion in different tissues. We have isolated and characterized a full-length cDNA clone for chicken N-cadherin and used this clone to transfect S180 mouse sarcoma cells that do not normally express N-cadherin. The transfected cells (S180cadN cells) expressed N-cadherin on their surfaces and resembled S180 cells transfected with L-CAM (S180L cells) in that at confluence they formed an epithelioid sheet and displayed a large increase in the number of adherens and gap junctions. In addition, N-cadherin in S180cadN cells, like L-CAM in S180L cells, accumulated at cellular boundaries where it was colocalized with cortical actin. In S180L cells and S180cadN cells, L-CAM and N-cadherin were seen at sites of adherens junctions but were not restricted to these areas. Adhesion mediated by either CAM was inhibited by treatment with cytochalasin D that disrupted the actin network of the transfected cells. Despite their known structural similarities, there was no evidence of interaction between L-CAM and N-cadherin.

Doubly transfected cells (S180L/cadN) also formed epithelioid sheets. In these cells, both N-cadherin and L-CAM colocalized at areas of cell contact and the presence of antibodies to both CAMs was required to disrupt the sheets of cells. Studies using divalent antibodies to localize each CAM at the cell surface or to perturb their distributions indicated that in the same cell there were no interactions between L-CAM and N-cadherin molecules.

These data suggest that the $Ca^{++}$-dependent CAMs are likely to play a critical role in the maintenance of epithelial structures and support a model for the segregation of epithelia based on differences in specificity of CAM mediated binding. They also provide further support for the so-called precedence hypothesis that proposes that expression and homophilic binding of CAMs are necessary for formation of junctional structures in epithelia.



CELL adhesion molecules (CAMs)[1] take part in fundamental morphogenetic events such as epithelial-mesenchymal transformation (EMT) and reorganization of epithelial boundaries. EMT (18, 35) is the reversible conversion of cells linked in epithelial sheets to loosely associated cells known as mesenchyme. Examples of EMT include the separation and migration of neural crest cells from the neural plate and the formation of the sclerotome in somites. Consistent with their role in cell linkage, the expression of CAMs at the cell surface decreases as mesenchyme is produced and increases when mesenchyme condenses (2). CAMs are also modulated at the cell surface when a contiguous homogeneous epithelium is reorganized into two epithelia that eventually segregate. This is seen, for example, during the differentiation of the neural plate (2, 38) and the differentiation of the otocyst (33). In such processes, the cells remain linked in epithelial sheets and do not undergo EMT. The epithelium initially expresses at least two CAMs, but, as the two epithelia segregate into different structures, the CAMs are differentially expressed in time and space.

Evidence has accumulated to suggest that $Ca^{++}$-dependent CAMs such as liver CAM (L-CAM) (11) and N-cadherin (16, 42) are necessary for the formation of epithelia and for the maintenance of cell junctional structures after they have formed (14, 15, 25). L-CAM was initially purified from embryonic chicken liver but is found in most nonneuronal epithelial cells (39). It is expressed at the surface of the cells as a glycoprotein of 124 kD that binds by a homophilic mechanism. L-CAM appears on the earliest embryonic cells to-

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; A-CAM, adherens junction-specific CAM; L-CAM, liver CAM; N-CAM, neural CAM; EMT, epithelial-mesenchymal transformation.

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gether with the neural CAM (N-CAM) and these primary CAMs are differentially expressed at a variety of cell borders at various sites of embryonic induction (2). The structurally related CAM, N-cadherin, was first characterized as a $Ca^{++}$-dependent CAM of the nervous system (16). Its distribution during development and in adult tissue (37) appears to be the same as the distribution of the adherens junction-specific CAM (A-CAM) (5) that was first isolated from chicken heart and lens (40, 43). The distribution of N-cadherin indicates that its differential expression may be associated with the formation of borders in various embryonic tissues (4, 37) as was demonstrated for N-CAM and L-CAM.

We have recently shown that the expression of L-CAM in S180 mouse sarcoma cells (which do not normally produce this CAM or N-cadherin) after transfection with cDNA induces a phenotypic change in these cells from a fibroblastic to an epithelioid phenotype. This alteration was accompanied by marked increases in the expression of adherens and gap junctions (25). We have now transfected S180 cells with the cDNAs for N-cadherin and L-CAM together in an effort to provide evidence for the role of two different CAMs in the reorganization of epithelia, and to analyze differences in their binding specificity to gain further insight into the role of CAMs during EMT.

Cells expressing N-cadherin (S180cadN) aggregated and underwent a phenotypic change similar to that observed for S180 cells expressing L-CAM (S180L). In both cases, aggregation was inhibited after disruption of microfilaments with cytochalasin D. S180L and S180cadN cells did not bind to each other and when mixed, they separated into distinct homogeneous collectives linked internally by gap junctions. In cells that were doubly transfected with cDNAs for L-CAM and N-cadherin, both molecules were active and accumulated at sites of cell contact, but they migrated independently on the cell surface. These results (*a*) generalize our previous work on L-CAM by showing that $Ca^{++}$-dependent CAMs can induce communicating junctions; (*b*) demonstrate that there is no significant interaction between chicken L-CAM and N-cadherin when they are located either on opposing cell membranes or on the same membrane; and (*c*) provide evidence that, in mixed populations, cells sharing a $Ca^{++}$-dependent CAM will selectively establish communicating junctions.

## Materials and Methods

### Recombinant DNA

A λgt10 12d embryonic chick brain library (kindly provided by Dr. H. Hanafusa, the Rockefeller University) was screened by nucleic acid hybridization (24) using degenerate DNA probes deduced from the $NH_2$-terminal amino acid sequence of N-cadherin (17). The Eco RI restriction fragments of the recombinant phages isolated were subcloned in Bluescript (Stratagene, La Jolla, CA) and sequenced by the dideoxynucleotide-chain termination method (34) using Sequenase enzyme (United States Biochemicals Corporation, Cleveland, OH). A full-length N-cadherin cDNA was constructed in Bluescript using standard procedures (24), and then recloned in the Bgl II site of pKSV10 vector (Pharmacia Fine Chemicals, Piscataway, NJ) downstream of the SV40 early promoters to give pKcadN 1853.

### Cell Culture and Transfections

S180 mouse sarcoma cells (6) (provided generously by Dr. Jean-Paul Thiery) were grown in DME supplemented with 15% FCS (Gibco Laboratories, Grand Island, NY) in tissue culture dishes (Corning Glass Works, Corning, NY) or on microscope slides coated with poly-L-lysine and human fibronectin (New York Blood Center, New York, NY).

S180 cells were cotransfected with pKcadN 1853 and pSV2neo (Pharmacia Fine Chemicals) vectors using the calcium phosphate method (3); similarly, S180 cells transfected with pSV2neo served as controls. Permanently transfected cell lines were selected in the presence of 0.4 mg/ml G418 (Gibco Laboratories) as previously described (8). Cells expressing both N-cadherin and L-CAM were obtained by cotransfecting S180L cells with pKcadN 1853 and pMSG (Pharmacia Fine Chemicals) vectors and selecting in gpt medium (27).

### Immunoblotting

SDS-PAGE (6%) and immunoblotting were done essentially as described previously (25) using anti-chicken L-CAM polyclonal antibodies plus [$^{125}$I]protein A for L-CAM. For N-cadherin, a polyclonal antibody directed against a synthetic peptide corresponding to amino acid residues $Asn^{12}$ to $Arg^{35}$ of N-cadherin or the anti-A-CAM monoclonal antibodies FA-5 (BioMakor, Rehovot, Israel) and ID-7.2.3 (Sigma Chemical Co., St. Louis, MO). These antibodies reacted specifically with N-cadherin. [$^{125}$I]sheep anti-mouse IgG (Amersham Corp., Arlington Heights, IL) was used as second antibody.

### Lactoperoxidase Radioiodination and Immunoprecipitation

Cells grown to confluence in 6-cm dishes were radioiodinated as described (9) using 1.5 mCi of Na $^{125}$I/dish. After washing four times in PBS, the cells were extracted in 10 mM Tris buffer, pH 8.8, 0.3% SDS, 0.1 mg/ml DNAse. The extract was diluted in 10 vol of PBS pH 7.4, 0.5% NP-40 and incubated with anti-A-CAM antibody FA-5 coupled to protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO) for 6 h at 4°C. The beads were washed in PBS and boiled in Laemmli sample buffer for 5 min. SDS-PAGE of immunoprecipitates was performed as previously described (25).

### Immunofluorescent Staining

Cell cultures were fixed in PBS, pH 7.4, containing 2.5% formaldehyde and 0.05% glutaraldehyde as described (8) and then stained. For N-cadherin staining, the anti-A-CAM monoclonal antibody FA-5 was added to the cells at a 1/1,000 dilution for 1 h, followed by the addition of biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) and rhodamine streptavidin conjugates (Molecular Probes, Eugene, OR). For N-cadherin and L-CAM double staining, anti-L-CAM and FA-5 antibodies were simultaneously added to cell cultures followed by the addition of biotinylated anti-mouse IgG, plus fluorescein goat anti-rabbit IgG conjugates (ICN Biomedicals Inc., Costa Mesa, CA) and then rhodamine streptavidin conjugates. For actin and N-cadherin double staining, cells were first permeabilized with 0.1% Triton X-100 in PBS for 15 min and stained as for N-cadherin alone, except that rhodamine phalloidin (dilution 1:10; Molecular Probes, Eugene, OR) was added to the first antibody, and rhodamine-streptavidin conjugates were replaced by fluorescein-streptavidin conjugates (Molecular Probes).

### Antibody Perturbation

Confluent cultures of transfected cells were incubated in culture medium with FA-5 monoclonal antibodies (1:50 dilution) and with Fab' fragments of anti-L-CAM antibodies (1 mg/ml) separately, or with both antibodies together at 37°C for 30 minutes and then fixed. To induce L-CAM patching, cells were incubated in the presence of divalent anti-L-CAM antibodies (0.1 mg/ml in the culture medium) at 37°C for 30 min and then fixed. To induce N-cadherin patching, cells were incubated with FA-5 antibodies (1:500 dilution in culture medium) and then with anti-mouse IgG, both at 37°C for 15 min, and were then fixed.

### Electron Microscopy

Preparation of samples were carried out as described (25). For immunoelectron microscopy, cell cultures were fixed and treated with primary antibodies as for immunofluorescent staining. FA-5 antibodies were revealed by rabbit anti-mouse IgG (ICN Biomedicals Inc.) followed by anti-rabbit immunoperoxidase (1:400 dilution for 30 min), and anti-L-CAM by anti-rabbit immunoperoxidase. Cells were then fixed in 1.5% glutaraldehyde in PBS

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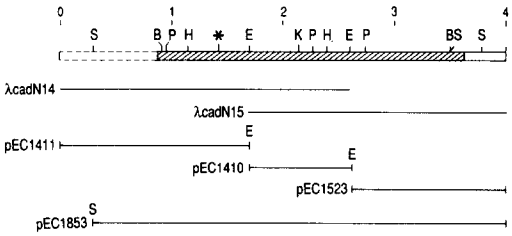


*Figure 1.* Structure of N-cadherin cDNA clones. Eco RI inserts contained in phages λcadN14 and λcadN15 were subcloned (pEC-1411, pEC1410, and pEC1523) and assembled in Bluescript to produce full length cDNA, pEC1853, which was inserted into the expression vector pKSV10 downstream of the SV40 early promoter. The top line shows the restriction map of the full-length cDNA:*B*, Bam HI; *E*, Eco RI; *H*, Hind III; *K*, Kpn I; *P*, Pst I; *S*, Sac I. *Dashed outline*, new 5′ untranslated sequences. *Hatched box*, open reading frame; *asterisk*, the position of the putative $NH_2$ terminus of the mature protein.

and washed in 50 mM of Tris buffer, pH 7.5. After development of peroxidase with diaminobenzidine and hydrogen peroxide (0.1%, 0.15%, 5 min in dark), the cells were washed several times in PBS, and treated with osmium tetroxide (1% in 0.1 M sodium cacodylate buffer, pH 7.2), and then with 1% uranyl acetate; they were dehydrated, embedded, and thin sections were prepared. Sections were viewed without counterstaining with an electron microscope.

### Cell Aggregation Assays

Aggregation assays were performed as described (13), except that cells ($2 \times 10^5$ in 600 μl medium) were shaken for 40 min in 24-well plates that had been coated with 1% agarose in PBS and equilibrated with aggregation medium (MEM or Spinners MEM without bicarbonate/20 mM Hepes). Cells were obtained from subconfluent monolayers by treating them with PBS containing 5 mM EDTA and 2% FBS.

### Dye Coupling Experiments

Cultures of $10^6$ cells, each containing S180L and S180 cadN cells, one of which had been labeled overnight with dil (3 μg/ml), were plated on 35-mm Corning tissue culture dishes in 2 ml DME/25 mM Hepes/15% FCS, overnight. Cultures were then mounted in a water-jacketed microscope stage that was adjusted to maintain the culture medium at 37–38°C. A group of confluent cells was selected and one cell was impaled with a glass micropipette (40–150 megohm resistance). Dye was injected for 1.5–2 min with 5 nA of hyperpolarizing current. Micropipettes were filled with 3% Lucifer yellow (Sigma Chemical Co.) and backfilled with 1 M $LiCl_2$. After injection, the culture was fixed for 30 min in 3.7% formaldehyde in PBS, 0.25 mM $CaCl_2$ at 37°C. Cells were fixed before photography (*a*) because the dye transfer in the monolayers was so fast and extensive that it would be undetectable after a fairly short time, and (*b*) so that we could mount the cells with an inhibitor of bleaching for photography. The culture was washed in PBS, mounted in MOWIOL-DABCO (20, 23), and the injected cells were photographed with a microscope (Axiophot; Carl E. Zeiss, Thornwood, NY) and Kodacolor 100 ISO film, using filter combination No. 09 to visualize the Lucifer yellow and filter combination No. 15 to visualize the dil. Phase illumination was used to photograph the field of cells.

## Results

### Isolation and Characterization of N-Cadherin cDNA

cDNA coding for the entire N-cadherin polypeptide was obtained from a λgt10 embryonic chick brain library by screening with degenerate DNA probes corresponding to the $NH_2$-terminal amino-acid sequence of N-cadherin (17). A recombinant phage, λcadN14, that contained a 1.7-kb insert was isolated and the insert was then used to screen the same library to give a second recombinant phage, λcadN15, (Fig. 1) that overlapped the 3′ end of the λcadN14 and extended 1.4-kb downstream. 90% of the sequence of three Eco RI inserts from these two clones included an open reading frame of ~2,700 bp that was identical to the N-cadherin cDNA sequence (17). The restriction map of the cDNA (Fig. 1) was identical to the restriction map of N-cadherin cDNA (17) and we concluded that this cDNA specified N-cadherin. The open reading frame was flanked by 0.3 kb of 3′ untranslated sequence containing a polyadenylation site, and by 0.9 kb of untranslated sequence upstream of the start codon. A com-

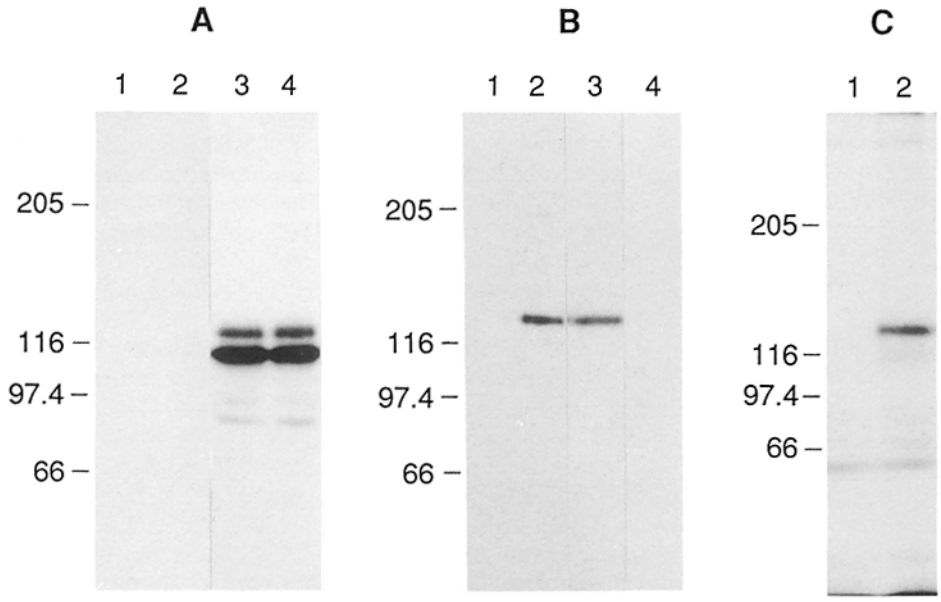


*Figure 2.* Expression of L-CAM and N-cadherin in transfected S180 cell lines. Extracts containing ~30 μg of total cell protein from S180 cells (*A*, lane *1* and *B*, lane *1*), S180 cells transfected with N-cadherin cDNA, S180-cadN cells (*A*, lane *2* and *B*, lane *2*), S180 cells transfected with both N-cadherin and L-CAM cDNAs, S180L/cadN cells (*A*, lane *3* and *B*, lane *3*), and S180 cells transfected with L-CAM cDNA, S180L cells (*A*, lane *4* and *B*, lane *4*) were loaded on a 6% polyacrylamide gel and immunoblotted with a polyclonal anti-L-CAM antibody (*A*) or with the monoclonal anti-A-CAM antibody FA-5 (*B*). *C*, Radioiodinated cell surface proteins from S180 (lane *1*) and S180-cadN (lane *2*) cells were immunoprecipitated as described in Materials and Methods and resolved on a 6% polyacrylamide gel.

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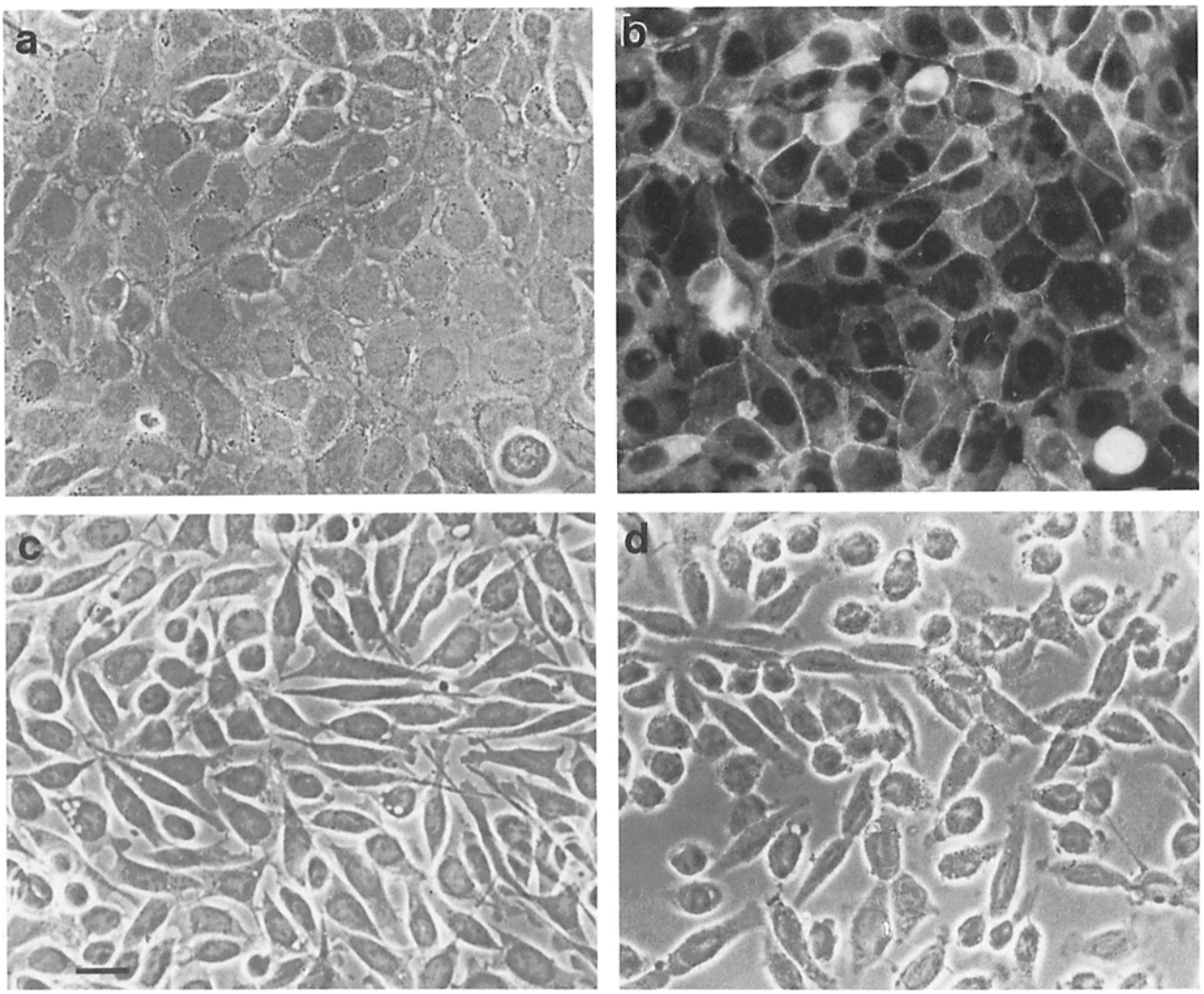


*Figure 3.* Changes in morphology in S180cadN cells. Monolayers of S180cadN cells were stained with monoclonal antibody FA-5 and fluorescent antibodies and examined under phase (*a*) or fluorescence microscopy (*b*). The epithelioid monolayers were disrupted after a 2-h incubation with monoclonal antibody FA-5 (*d*) and resembled untransfected S180 cells (*c*). Bar, 10 μm.

plete cDNA, pEC1853, was constructed in Bluescript by the juxtaposition of the three Eco RI inserts.

### *Transfection of N-cadherin cDNA into S180 Cells*

An expression vector containing the entire coding sequence of N-cadherin was constructed by cloning the Sst I/Xba I insert of pEC1853 into the Bgl II site of pKSV10 downstream of the SV40 early promoter. The resulting plasmid, pKcadN-1853, was co-transfected into S180 cells together with pSV2neo. Three independent, permanently transfected cell lines were isolated.

The cell lines expressed the expected 135-kD polypeptide that was recognized in Western blots by monoclonal antibody FA-5 (Fig. 2 *B*, lane *2*) and by another monoclonal anti-A-CAM antibody ID-7.2.3 (42), as well as by a polyclonal antibody directed against a synthetic peptide corresponding to amino acid residues $Asn^{12}$ to $Arg^{35}$ of N-cadherin (not shown). These same antibodies recognized a weak but detectable 180-kD polypeptide in extracts of transfected cells, or chicken brain and chicken heart but not in extracts of untransfected cells. The nature of this component and its relation to N-cadherin is unknown and is currently being investigated. Pulse chase experiments indicated that it was not a precursor of the 135-kD peptide, and immunoprecipitation of radioiodinated surface proteins of S180cadN cells showed that only the 135-kD peptide was expressed at the surface of these cells (Fig. 2 *C*, lane *2*). The observations that our sequence is identical to that of N-cadherin and that the protein product reacts with monoclonal antibodies to A-CAM (40) support the notion that N-cadherin and A-CAM are the same molecule (5).

To study possible interactions between N-cadherin and L-CAM (5) expressed in the same cell, S180 cells already expressing chicken L-CAM (S180L; reference 25) were transfected with pKcadN 1853 as described in Materials and Methods. In immunoblots of boiling SDS extracts of S180L cells, L-CAM appeared as a doublet (Fig. 2 *A*, lane *4*). The major band, which contained roughly 80% of the counts, comigrated with chicken liver L-CAM. The minor band had a higher $M_r$, which was identical to that established for the

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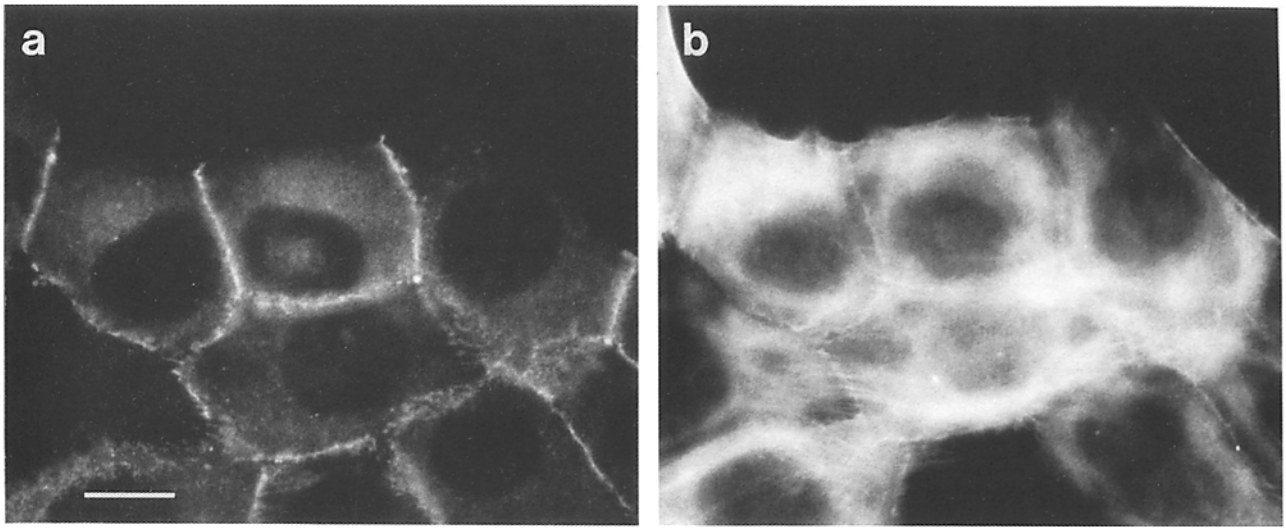


*Figure 4.* Colocalization of actin and N-cadherin at regions of contact between S180cadN cells. Subconfluent cultures of S180cadN cells were fixed and stained with the monoclonal antibody FA-5 for N-cadherin (*a*) and with rhodamine phalloidin for actin (*b*). N-cadherin is highly concentrated at regions of contact between cells where it colocalizes with cortical actin. Bar, 5 μm.

L-CAM precursor in primary hepatocyte cultures (36). This suggested that the minor band was a relatively long lived L-CAM precursor. Pulse chase analyses of immunoprecipitates of S180 cell extracts were consistent with this interpretation (S. H. Jaffe, B. A. Cunningham and G. M. Edelman, unpublished observations). Doubly transfected clonal cell lines were shown to express both L-CAM (Fig. 2 *A*, lane *3*) and N-cadherin (Fig. 2 *B*, lane *3*) by immunoelectrophoresis. As discussed below, the two molecules behaved independently when expressed within the same cell.

### *Phenotypic Changes Induced by N-cadherin Expression*

Untransfected S180 cells grew as loose, round and spindle shaped cells (Fig. 3 *c*) whereas S180cadN cells were flatter and bound to each other, forming colonies of epithelioid cells with polygonal boundaries (Fig. 3 *a*). When plated on a polylysine plus fibronectin substrate, confluent S180cadN monolayers resembled confluent S180L monolayers; when plated on uncoated tissue culture dishes, S180cadN epithelioid sheets were less uniform than those formed by S180L cells. N-cadherin accumulated at areas of contact between S180cadN cells, with little staining at the extrajunctional surfaces (Fig. 3 *b*). When these cells were incubated with monoclonal antibody FA-5, the transfected cells (Fig. 3 *d*) dissociated and resembled parent untransfected cells (Fig. 3 *c*). A similar disruption of cell contacts was caused by removal of $Ca^{++}$ from the medium (data not shown).

As observed for L-CAM in S180L cells (25), N-cadherin at regions of cell contact between S180cadN cells was apparently colocalized with accumulations of cortical actin (Fig. 4). In addition, cytochalasin D inhibited L-CAM and N-cadherin mediated aggregation (Table I). At the concentrations used here, cytochalasin D disrupted actin networks but did not cause bound cells to dissociate or disrupt actin bundles at boundaries already existing between transfected cells. This was observed previously for S180L cells. In contrast to the effect of cytochalasin D, neither nocodazole (which disrupts microtubules) nor azide had any effect.

### *Increase in Adherens Junctions and Gap Junction–like Structures*

The cultured S180cadN cells had increased numbers of adhe-

*Table I. Aggregation of S180cadN Cells and S180L Cells*

| | Aggregation medium | | | | | |
|---|---|---|---|---|---|---|
| Cells | MEM | SMEM | +Antibodies | +Cytochalasin D | +Nocodazole | +Azide |
| S180 | 28 ± 2 | 12 ± 2 | 30 ± 2 | 33 ± 1 | 29 ± 2 | 24 ($n$ = 1) |
| S180cadN | 48 ± 2 | 12 ± 2 | 33 ± 3 | 39 ± 6 | 48 ($n$ = 1) | 48 ($n$ = 1) |
| S180L | 70 ± 2 | 15 ± 2 | 18 ± 2 | 44 ± 3 | 64 ± 1 | 63 ($n$ = 1) |

Aggregation assays were carried out as described in Materials and Methods. Cells were incubated with reagents for 20 min, before initiation of assay. Concentrations were as follows: 300 μg/ml $F_{ab'}$ fragments of polyclonal anti-L-CAM IgG, 1:100 dilution of monoclonal FA-5 anti-A-CAM ascites fluid; 0.2 μg/ml cytochalasin D, 1 μg/ml nocodazole, and 10 mM azide. Numbers represent percentage of aggregation after 40 min and were determined as described; results are averages ± SEM ($n$ = 3–5, except where noted otherwise). Anti-A-CAM and anti-L-CAM antibodies were used for S180cadN and S180L cells, respectively; no antibodies were used for S180 cells.

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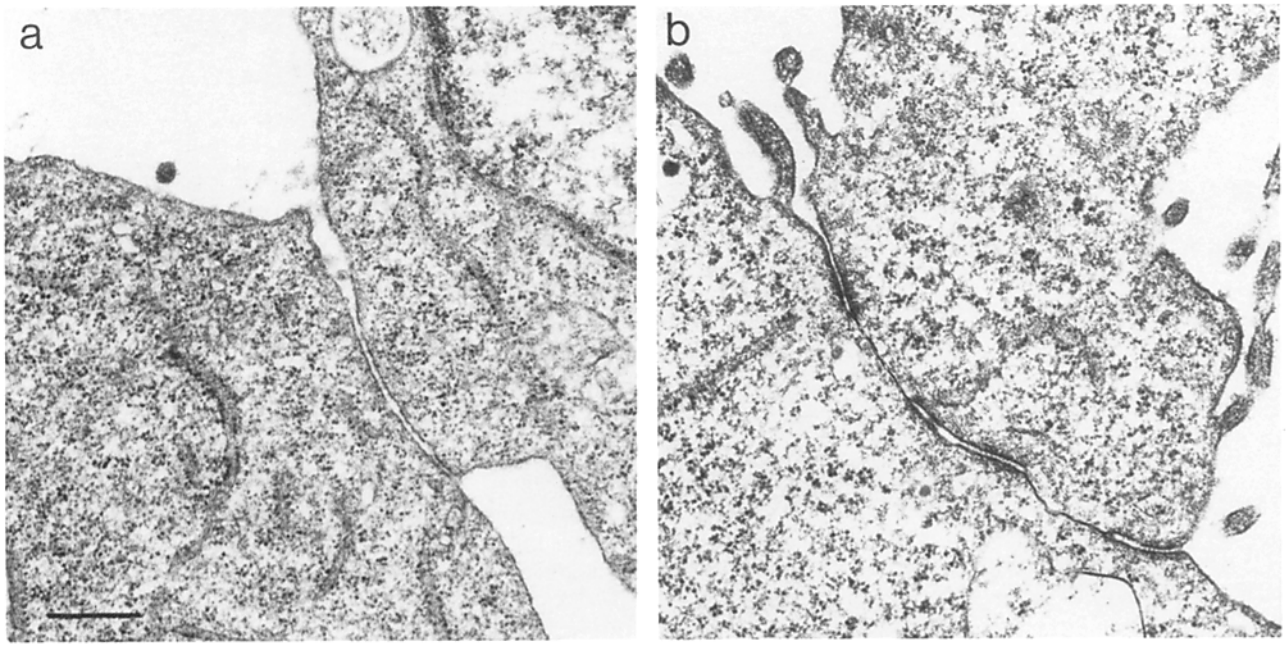


*Figure 5.* Cellular junctions in confluent S180cadN cells. Electron micrographs of thin sections of S180 cells (*a*) and S180 cadN cells (*b*). S180cadN cells have many adherens junctions and gap junctions at areas of cell contact and the two types of junctions are often seen together in clusters. Bar, 0.5 $\mu$m.

rens junctions and gap junction–like structures as seen previously for S180L cells (25). Electron micrographs of sections of S180 cells showed very few regions resembling adherens and gap junctions (Fig. 5 *a*). In contrast, numerous extended and characteristic adherens junctions, associated with dense cytoplasmic accumulations, were seen in S180cadN cells (Fig. 5 *b*). These junctions were often intercalated with gap junction–like structures, distinguishable as directly apposed membranes (12).

Quantitative analyses (Table II) showed that, relative to untransfected controls, there was a fivefold increase in the length of apposed membranes, a threefold increase in the number of adherens junctions per unit length, and a 50-fold increase in the overall length of gap junction–like structures. Complementing these observations, dye coupling experiments provided strong evidence that transfection of N-cadherin induced the formation of communicating junctions (see the Dye Transfer section). The percentage of apposed membrane in S180cadN cells was only 30% higher than in S180cadN cells grown in the presence of monoclonal FA-5 antibody, a smaller difference than that suggested by Fig. 3 (*a* and *d*). This apparent discrepancy between the quantitative data presented in Table I and the qualitative impression conveyed by Fig. 3 (*a* and *d*) was probably because of differences in the protocols. For the quantitative data obtained from electron micrographs, cultures were incubated with antibodies for 30 min; for the light micrographs of Fig. 3, cultures were treated with antibodies for 2 h. More importantly, although the numbers of apposed membrane for cultures of S180cadN cells incubated with and without FA-5 antibody were closer than may be expected, the numbers of gap junction–like structures and adherens junctions were sharply lower for the cultures incubated with FA-5 antibody. The increase in the number of adherens and gap junction–like structures in S180cadN was one-third lower than that observed in S180L cells, consistent with the observed differences in aggregation observed for S180cadN and S180L cells (Table I). It is not clear whether these result from differences in levels of expression or in binding strengths of N-cadherin and L-CAM; adequate analysis will require devising a thermodynamic assay for CAM binding.

Immunoperoxidase electron microscopy (Fig. 6) was used to localize N-cadherin and L-CAM in the transfected cells. L-CAM was generally present over the entire surface of S180L cells, with accumulations in those areas resembling adherens junctions (Fig. 6, *a* and *a'*). Similarly, N-cadherin was detected over the surface of S180cadN cells, with even more intense accumulations at adherens junctions (Fig. 6, *b* and *b'*). In control experiments, anti–L-CAM antibodies did not stain S180cadN cells (Fig. 6 *e*) and monoclonal antibody FA-5 detected no components in S180L cells (Fig. 6 *f*).

*Table II. Formation of Adherens and Gap Junction–like Structures in S180 Cells Expressing N-cadherin*

| Cells | Total membranes measured ($\mu m$) | Percentage of apposed membrane | Adherens junctions* | Gap junction–like structures‡ |
|---|---|---|---|---|
| S180 | 2,030 | 6.6 | 1.5 | 0.2 |
| S180cadN | 1,602 | 29.8 | 9.2 | 9.4 |
| S180cadN (+ anti-A-CAM) | 1,046 | 23.0 | 3.0 | 0.1 |

* Numbers of junctions with 10–20-$\mu$m spacing and dense cytoplasmic inclusions/100 $\mu$m of apposed membrane.
‡ Microns of directly apposed membrane/100 $\mu$m of apposed membrane.

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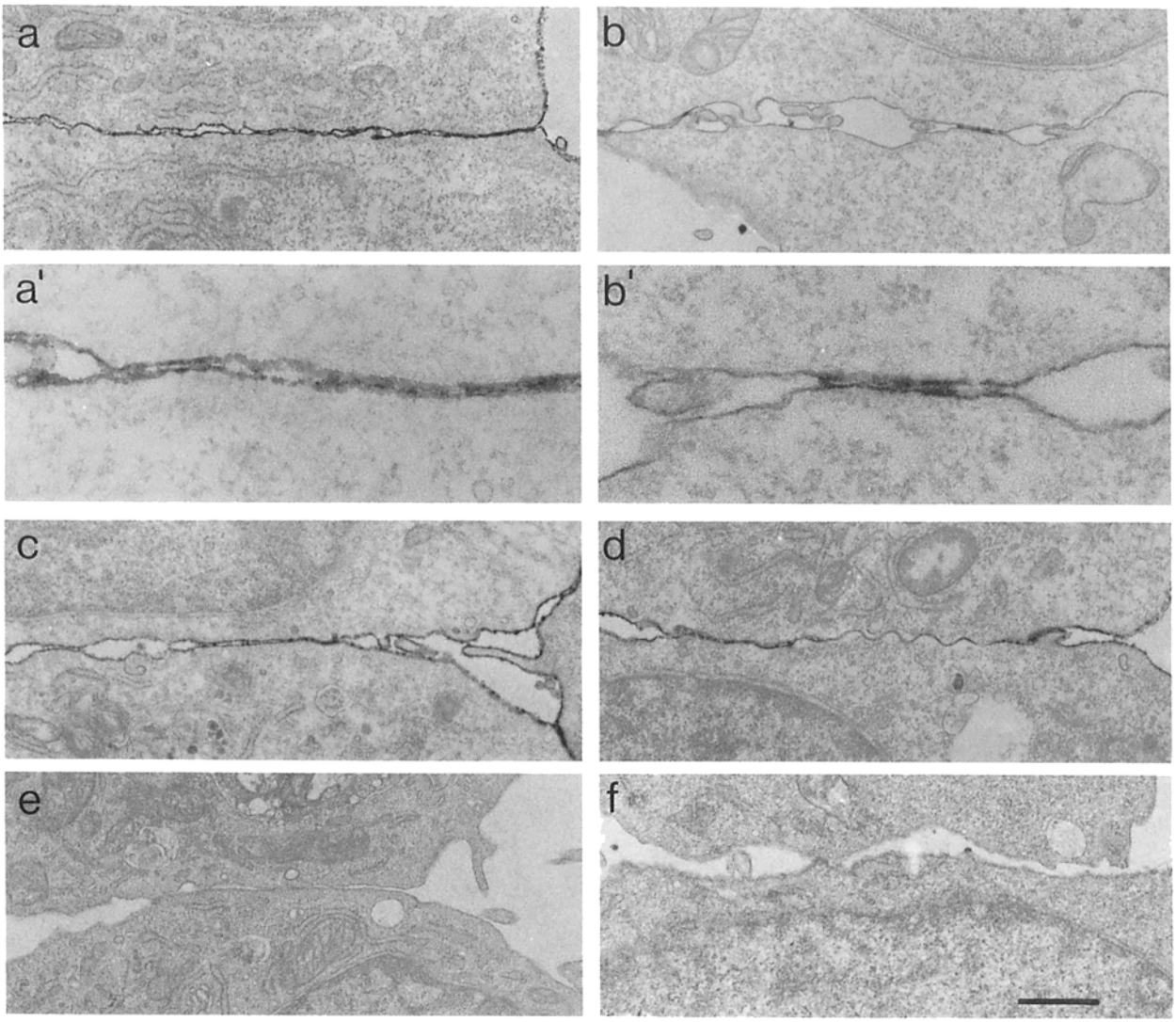


*Figure 6.* Ultrastructural localization of N-cadherin and L-CAM in transfected S180 cells. Cultures of S180L (*a*, *a'*, and *f*), S180cadN (*b*, *b'*, and *e*), and S180L/cadN were processed for immunoperoxidase labeling after incubation with polyclonal anti-L-CAM IgG (*a*, *a'*, *c*, and *e*) or monoclonal FA-5 antibodies (*b*, *b'*, *d*, and *f*), as described in Materials and Methods. Labeling is recognized as a dark deposit at the cell membrane. Micrographs *a'* and *b'* are higher magnification views of regions in micrographs *a* and *b*, respectively. Bar, (*a*, *b*, *c*, *d*, *e*, and *f*) 1 μm; (*a'* and *b'*) 250 nm.

### *Independent Behavior of N-cadherin and L-CAM Expressed within the Same Cells*

When L-CAM and N-cadherin were coexpressed in the same cell (S180L/cadN cells), both were more concentrated at cell boundaries than elsewhere on the cell surfaces (Fig. 7, *a* and *b*). This difference was more apparent for N-cadherin than for L-CAM. At the ultrastructural level, the distributions of L-CAM (Fig. 6 *c*) and N-cadherin (Fig. 6 *d*) in doubly transfected cells were similar to the distribution of these molecules in singly transfected cells (Fig. 6, *a* and *a'*).

To test for possible interactions between N-cadherin and L-CAM on the same cell (*cis* interactions), S180L/cadN cells were treated with divalent anti-L-CAM antibodies to induced micropatching of L-CAM. The formation of such patches removed L-CAM from the regions of cell contact (Fig. 7 *c*), but did not affect the localization of N-cadherin, which remained at areas of cell contact (Fig. 7 *d*). Similarly, treatment of S180L/cadN cells with the monoclonal antibody FA-5, followed by divalent anti-mouse IgG, induced formation of N-cadherin patches (Fig. 7 *f*) without affecting the distribution of L-CAM (Fig. 7 *e*).

Antibodies to both CAMs were required to disrupt cell-cell interactions in doubly transfected S180L/cadN cells (Fig. 8). Fab' fragments from anti-L-CAM antibodies (Fig. 8 *b*) or FA-5 monoclonal antibody (Fig. 8 *c*) added separately to the culture medium for 2 h did not disrupt the cell contacts, although under the same conditions, they were able to disrupt layers of S180L and S180cadN cells, respectively. However, when both antibodies were added together, S180L/cadN cells dissociated rapidly (Fig. 8 *d*), consistent with the conclusion that homophilic interactions of one kind of CAM were sufficient to maintain stable cell-cell interactions in the sheets.

### *Binding in Various Heterotypic Mixtures*

In co-cultures, S180L and S180cadN cells (Fig. 9, *a–c*) did

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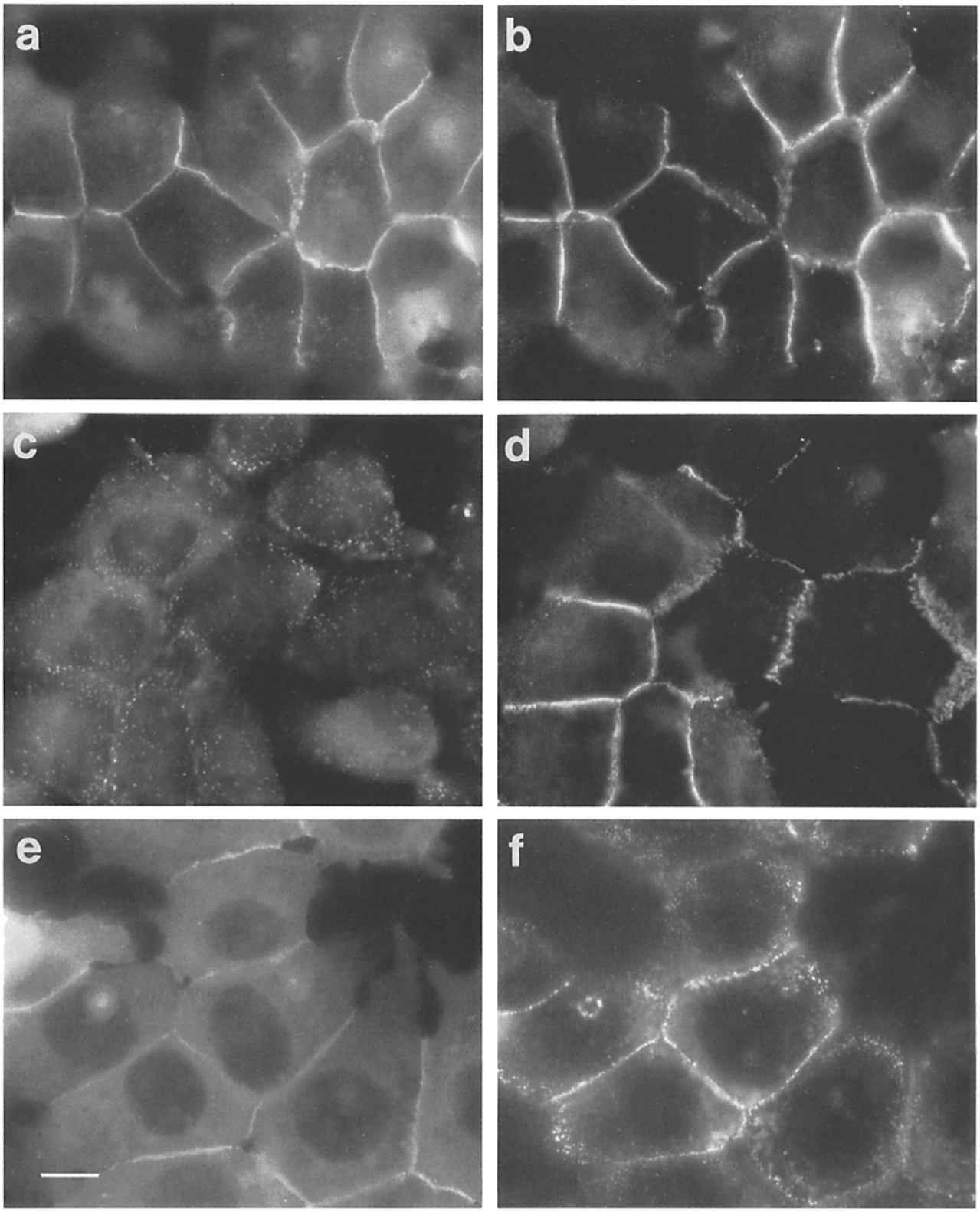


*Figure 7.* Distribution of L-CAM and N-cadherin in doubly transfected S180L/cadN cells. Before fixation, cultures were incubated either in culture medium alone (*a* and *b*), or in the presence of divalent anti-L-CAM antibodies or of FA-5 monoclonal antibody and biotinylated anti-mouse IgG (*e* and *f*). Each culture was then fixed and stained for both L-CAM (*a*, *c*, and *e*) and N-cadherin (*b*, *d*, and *f*) and analyzed through immunofluorescence microscopy. In untreated cells L-CAM (*a*) and N-cadherin (*b*) are colocalized at regions of contact between cells. Anti-L-CAM induced patching of L-CAM epitopes (*c*) that moved out of the regions of cell contact but did not affect N-cadherin distribution (*d*). Similarly, patching of N-cadherin antibody with FA-5 (*f*) did not affect L-CAM staining (*e*). Bar, 5 μm.

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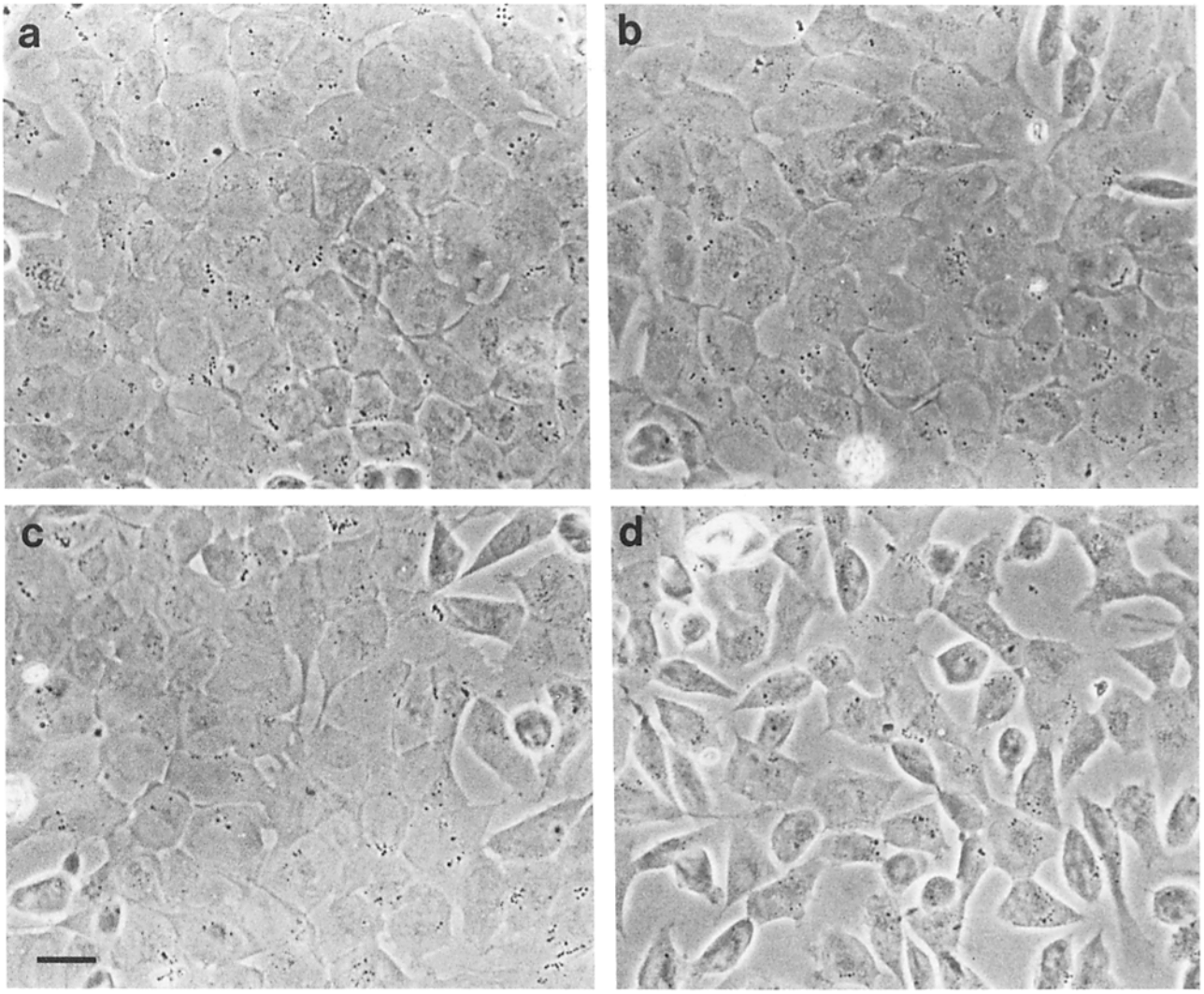


*Figure 8.* Effect of specific antibodies on the morphology of doubly transfected S180L/cadN cells. Cells were incubated with nonimmune rabbit Fab' fragments (*a*), anti-L-CAM Fab' fragments (*b*), FA-5 monoclonal anti-A-CAM antibodies (*c*), or with both anti-CAM antibodies (*d*) for 2 h at 37°C and then fixed. Anti-L-CAM and anti-A-CAM added together (*d*) disrupted the layer of cells (S180L/cadN), whereas each antibody by itself (*b* and *c*) had little or no effect. Bar, 10 μm.

not appear to interact with each other. In the few cases where cells of the different types were in close proximity, neither L-CAM (Fig. 9 *b*) nor N-cadherin (Fig. 9 *c*) was present at the borders between the cells (Fig. 9, *a–c*).

When doubly transfected S180L/cadN cells were co-cultured with S180L cells, (Fig. 9, *d–f*), L-CAM (Fig. 9 *e*) was distributed evenly over the boundaries between both cell populations. The N-cadherin on S180L/cadN cells (Fig. 9 *f*) was concentrated only at boundaries between S180L/cadN cells and other S180L/cadN cells and was excluded from boundaries between S180L/cadN and S180L cells. Comparable results were obtained in co-cultures of S180L/cadN with S180cadN cells (Fig. 9, *g–i*); i.e., N-cadherin (Fig. 9 *i*) was present at all boundaries and L-CAM (Fig. 9 *h*) was found only at those boundaries between cells expressing both CAMs. These results are consistent with those implying the independent behavior of each CAM in a given cell and also with the separate homophilic specificities of the two molecules.

### *Dye Coupling*

To investigate whether gap junctions could form between S180cadN cells, single cells in subconfluent cultures of S180cadN cells were injected with Lucifer yellow and neighboring cells were analyzed to determine dye coupling. As previously observed for transfected S180L cells (25), in most cases there was a clear spread of the dye to several cells in the vicinity of the injected cell (Fig. 10). When cell density was $1.8 \times 10^5$ cells/cm$^2$ there were a minimum of $17 \pm 6$ cells coupled to the injected cell. The coupling is probably more extensive than these numbers indicate because the dye intensity decreases smoothly with distance from the injected cell to barely detectable levels in cells 3–4 places removed from the injection site. The lack of a discrete edge to the injected dye intensity suggests that dye transferred to more cells at a level below visible detection limits. In the cases where no dye coupling was observed, the injected cell had been lifted by the micropipette.

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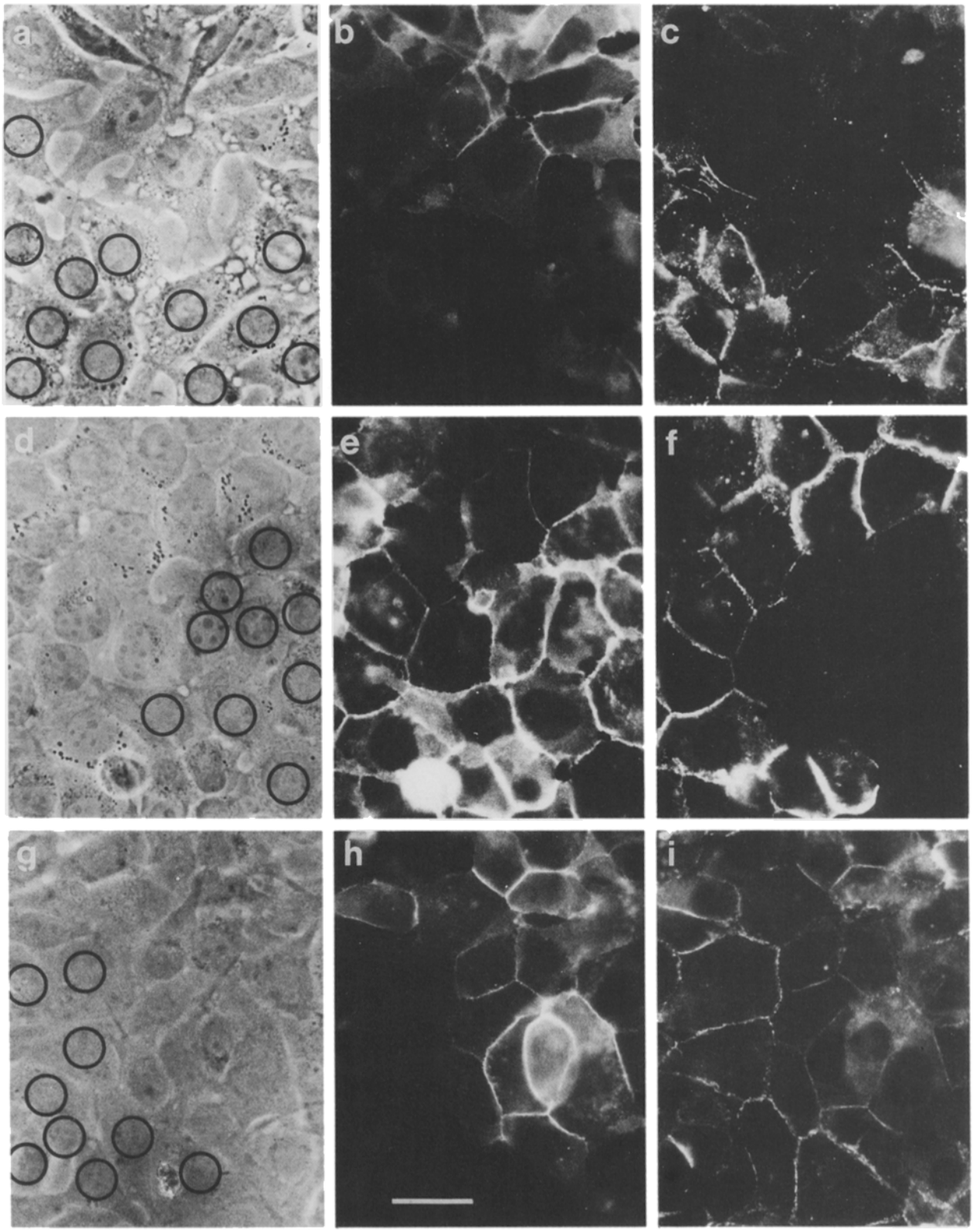


*Figure 9.* Co-culture of S180cadN cells with S180L cells and with S180L/cadN cells. S180cadN cells were co-cultured with S180L cells (*a*, *b*, and *c*) and doubly transfected S180L/cadN cells with S180L cells (*d*, *e*, and *f*) or with S180cadN cells, (*g*, *h*, and *i*) on a glass substrate coated with polylysine and fibronectin and grown for 2 d. Confluent cultures were fixed and stained for L-CAM (*b*, *e*, and *h*) and N-cadherin (*c*, *f*, and *i*). To help distinguish in each case the two types of cells, S180cadN cells (*a* and *c*) and S180L cells (*b*) were marked with circles in the phase micrographs. Bar, 10 $\mu$m.

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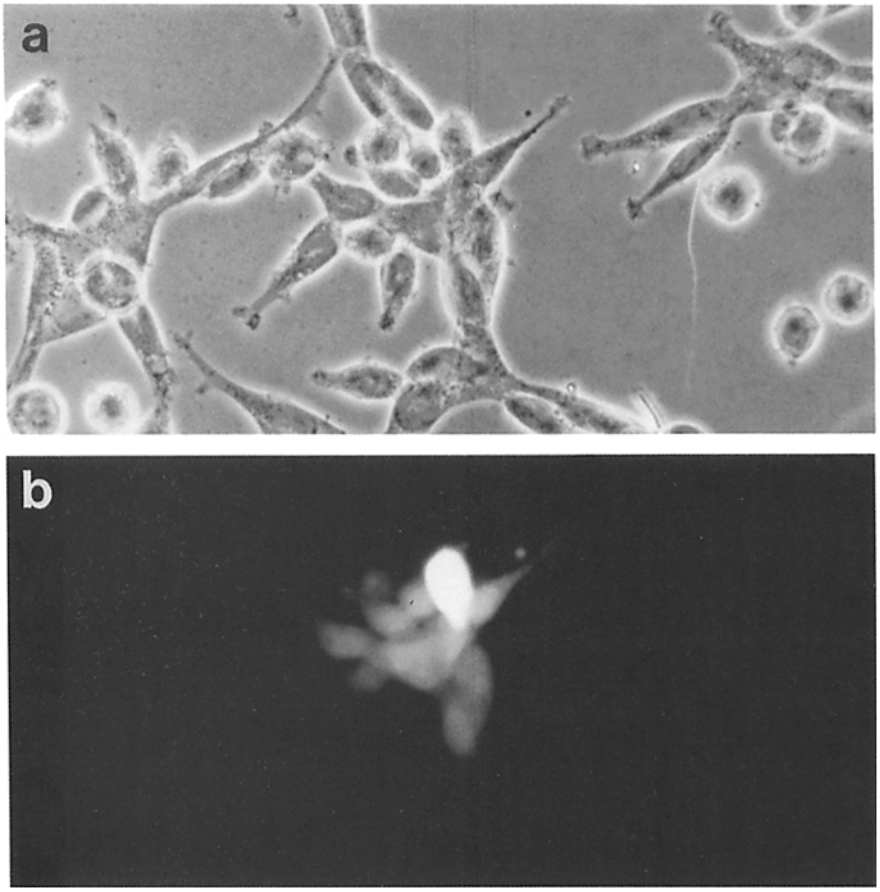


*Figure 10.* Dye coupling of S180cadN cells. (*a*) Phase illumination of field of cells. (*b*) Same field as in *a* viewed with fluorescent illumination to visualize the Lucifer yellow injected into one cell. The dye has spread extensively to the group of cells that are mutually adherent.

When subconfluent untransfected S180 cells were injected in cultures with a cell density of $1.9 \times 10^5$ cells/cm$^2$ dye coupling was much more restricted. An average of $5 \pm 2.3$ cells were coupled to the injected cell. Moreover, the boundaries of the injection were clearly delineated, indicating that all the coupled cells were visualized, and most of the coupled cells were nearest neighbors to the injected cell. This result is consistent with the ultrastructural observations in this and previous work (25), where a few gap junctions were found among S180 cells; slightly less dye coupling was detected between cells in the previous work, possibly because temperature and pH of the cultures were less well controlled than in the present experiments.

When co-cultured at subconfluency, S180L and S180cadN cells often formed mixed colonies of cells. The two different cell types were distinguished by labeling either S180L or S180cadN with the lipid stain diI. Single cells were injected with Lucifer yellow and the cultures were then evaluated for dye transfer; any injections into colonies that consisted exclusively of one cell type were ignored. Of the eight injections into mixed colonies, five had dye transfer exclusively to cells that expressed the same CAM as the injected cell. In the other three cases, only a single cell of a different type from the injected cell contained dye, while 5, 17, and 24 cells of the same type contained dye (Fig. 11). We also observed a similar amount of coupling between untransfected S180 cells and S180L cells in co-culture (data not shown).

Combined, these results indicated that S180L cells were dye-coupled with S180L cells, S180cadN cells were coupled with S180cadN cells, and that the frequency of the occasional coupling between S180L and S180cadN could not be distinguished from the "background" coupling between untransfected S180 cells.

## Discussion

The transfection of cell lines with cDNAs coding for particular cell adhesion molecules of different specificity provides a powerful system for the study of CAM function, particularly the effect of CAM expression on the phenotype and behavior of interacting cells. This approach has been used to confirm that CAMs can bind cells via homophilic mechanisms and that CAM mediated adhesion can lead to other cellular changes (8, 17, 25, 29, 30). These include changes in cell shape and also the expression of cellular junctions that are related to their function in vivo (25).

Using this approach in the present study, we were able to show that: (*a*) the expression of N-cadherin in S180 mouse sarcoma cells caused changes in cell phenotype similar to those obtained when these cells are transfected with L-CAM, providing further evidence for the role of epithelial CAMs in EMT. (*b*) There was no specific interaction of S180cadN and S180L cells in cultures, as is consistent with the results of cell adhesion assays (10). (*c*) In such adhesion assays, adhesion between cells expressing either of the CAMs was inhibited by treatment with cytochalasin D. (*d*) An increased number of adherens and gap junctions was formed in epithelioid sheets of S180cadN cells as a result of

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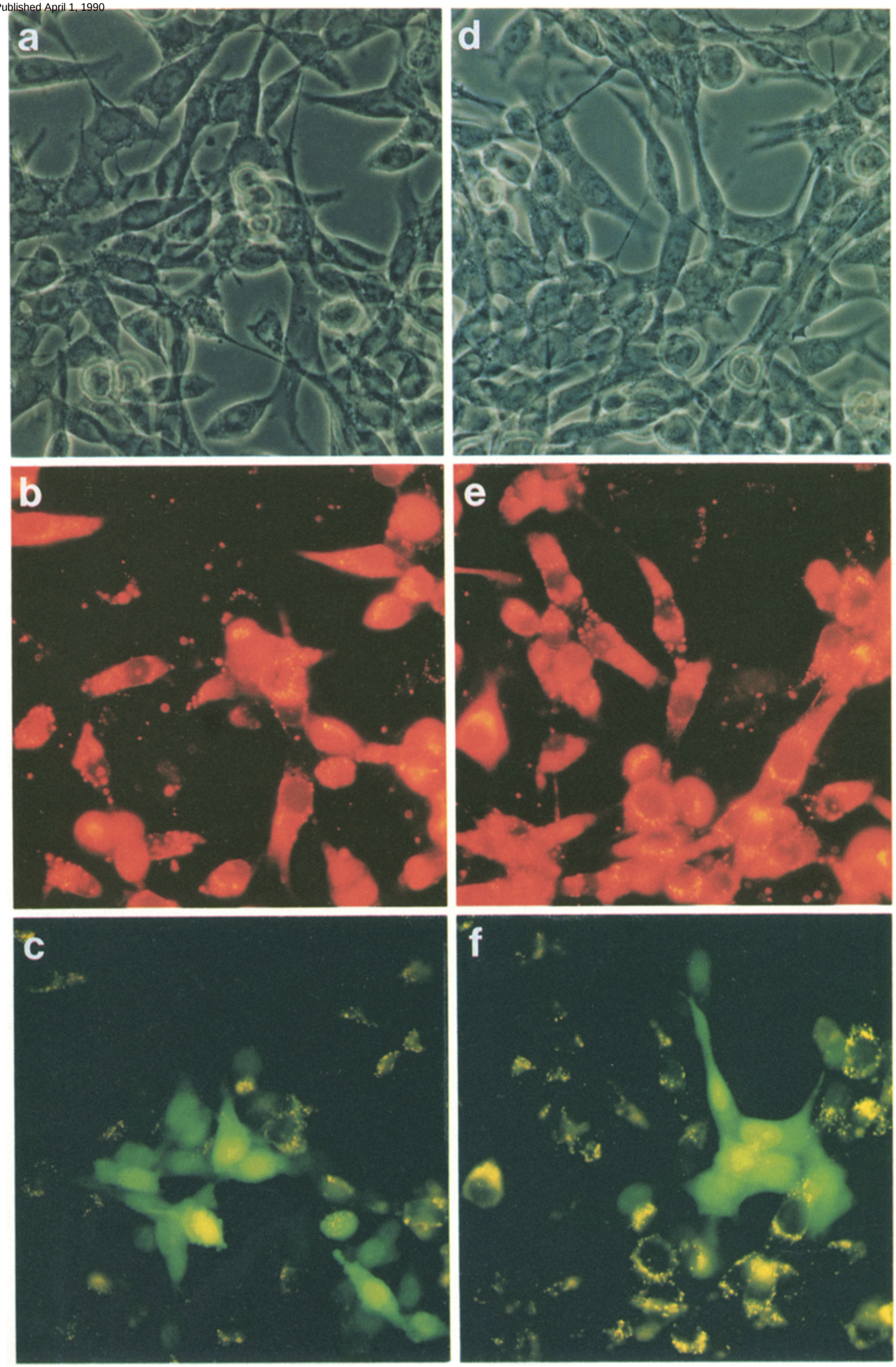

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homophilic binding of N-cadherin. These junctions sharply decreased in number when anti-A-CAM antibodies disrupted the sheets with a return to a cell shape resembling that of untransfected S180 cells. The increase in the number of gap junctions was accompanied by an increase of dye coupling among S180cadN cells. When S180L cells were cocultured with S180cadN cells, high levels of dye coupling were observed among S180cadN cells, but not between S180L and S180cadN cells. (*e*) In doubly transfected cells, the interactions occurred independently between each CAM (L-CAM and N-cadherin). Such cells interacted with singly transfected cells only by the shared CAM. Furthermore, only one CAM was sufficient to link doubly transfected cells; antibodies to both CAMs were necessary to disrupt sheets of such cells. (*f*) No *cis* interactions were found between the two CAMs, as shown by independent patching of each CAM in doubly transfected cells that was induced by appropriately specific antibodies.

At confluence, S180cadN cells had an epithelioid morphology with an increased number of adherens and gap junctions, suggesting that modulation of N-cadherin expression and cell binding can promote the formation of adherens junctions in vivo. This change has previously been shown to occur in S180L cells (25) and thus obviously does not depend on CAM specificity but rather on CAM linkage. We have shown here by immunoelectron microscopy that although L-CAM and N-cadherin are present at sites of adherens junctions, they were not restricted to adherens junctions in transfected S180 cells. In cells normally expressing A-CAM (N-cadherin), the molecule accumulates at adherens junctions (41, 42), which are in turn associated with actin bundles. Consistent with these observations, we saw an accumulation of N-cadherin and cortical actin at regions of contact between S180cadN cells. These results, together with those previously obtained for L-CAM (25) provide further support for the precedence hypothesis (7), which proposes that the linkage of cells by CAMs is a necessary event for the extensive expression of junctional structures. Transient gap junction formation (even with coupling) can occur rarely, however, with cells that are in proximity and not linked by any known CAM.

The inhibition of the aggregation of S180cadN cells and S180L cells by cytochalasin D suggests that the actin network plays an important role in L-CAM/L-CAM and N-cadherin/N-cadherin interactions. Direct or indirect interactions of the cytoplasmic domains of these molecules with the actin network may be needed for initial phases of cooperative CAM binding as well as for subsequent events including the formation of junctions that further stabilize CAM interactions. However, as suggested by the fact that cytochalasin D does not disrupt S180cadN and S180L cells that are already bound, the role of the actin network in the maintenance of these interactions may be minimal. Recent data (28, 31; Jaffe, S. H., B. A. Cunningham, and G. M. Edelman, unpublished results) suggest that the carboxyl terminal 50 amino acids of L-CAM are necessary for interaction of the cytoplasmic portion of L-CAM with cytoskeletal components. Because of the strong homology among the members of the $Ca^{++}$-dependent CAM family, especially in their cytoplasmic domains (19), other $Ca^{++}$-dependent CAMs probably have similar cytoplasmic interactions. It has also been found that the $Ca^{++}$-independent CAM, N-CAM, can interact with a known element of the cytoskeleton fodrin (32), further emphasizing the general importance to different CAM functions of such interactions.

There appeared to be little or no *cis* or *trans* interaction between the two CAMs as studied in the present investigation. Recent studies with mouse CAMs (26) also indicate that cells that express N-cadherin do not bind to cells that express L-CAM (E-cadherin). Furthermore, cell sorting experiments indicate that these cells form separate collectives of cells linked respectively by each CAM and showing discrete boundaries (10). Previous work had raised the possibility that L-CAM and N-cadherin might interact with each other (43). As shown here in the patching studies, no evidence for *cis* interactions was obtained, and the binding and distribution of N-cadherin and L-CAM expressed on the same cell were independent. Thus, the binding effects of CAMs of different specificity are likely to be additive; when redistribution occurs, each CAM can act to link that part of the cell surface to another cell in an independent fashion.

These observations and conclusions allow us to suggest a model for how the separate expression of CAMs of different specificity in vivo can lead to the reorganization of epithelia, using L-CAM and N-cadherin as examples. At early stages of induction a tissue may express both L-CAM and N-cadherin (a situation which is known to occur in vivo) each of which separately serves to link cells together to maintain a continuous epithelial structure. As differentiation proceeds, however, the CAMs may be differentially expressed at different cellular locations on the same sheet. Two collectives of cells would then segregate giving rise to two distinct and adjacent epithelia without any gross movements of the kind seen in EMT. Of course, other CAMs may independently be expressed in such sheets. The presence at the cell surface of at least one CAM would ensure the maintenance of an epithelial structure. The conversion of a unique epithelium into two separate collectives of cells may in turn allow for a differential response of the two epithelia to further induction events. Of course, appropriate functional changes would be expected to accompany segregation.

Such a model is not restricted to two CAMs of different specificities only and it may be extended to a variety of different CAMs, albeit with increased complexity. The central notion is that border formation in adjoining epithelia is likely to be derived from differences in both the specificity and sur-



*Figure 11.* Dye coupling in mixed cultures of S180L and S180cadN cells. (*a* and *d*) Phase illumination of two different injection sites. (*b* and *e*) Illumination in the rhodamine channel to visualize the dil cell marker, present in S180cadN cells (*b*) and in S180L cells (*e*). (*c* and *f*) Illumination in the fluorescein channel to visualize the Lucifer yellow, which produces a green fluorescence with this filter combination, and the dil, which produces a yellow fluorescence with this combination. The Lucifer fluorescence is mainly found in cells that express the same CAM. Cells in frame *c* pulled apart after injection.

face concentration of various CAMs (10), a hypothesis that can be tested further by using cDNAs constructed with inducible promoters to transfect cells in vitro.

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