Antibodies to liver cell adhesion molecule perturb inductive interactions and alter feather pattern and structure

(embryonic induction/skin histogenesis/pattern formation/morphogenesis)

W. J. GALLIN, C.-M. CHUONG, L. H. FINKEL, AND G. M. EDELMAN

The Rockefeller University, 1230 York Avenue, New York, NY 10021

ABSTRACT   Cell adhesion molecules (CAMs) may act as regulators of morphogenesis by constraining cell motion, forming borders, and controlling intercellular communications that lead to embryonic induction. This postulated causal role of CAMs in inductive events was tested here in an *in vitro* system of feather induction. In the developing chicken skin, an ectodermal sheet of epithelium interacts with mesodermal cell collectives to form more or less circular feather germs arranged in a hexagonal pattern. Cells of the epidermal epithelium are linked by liver CAM (L-CAM) and mesodermal cells in dermal condensations are linked by neural CAM (N-CAM); neither of these CAMs links cells in one tissue of this inductive couple to cells in the other. After perturbation of the L-CAM linkage in epidermis by antibodies to L-CAM, nonhexagonal striped patterns of dermal condensations were observed in culture. The stripes did not follow straight lines but meandered in lateral and oblique directions. Histological examination of the perturbed tissues showed extensive changes in dermal cell density distributions. After 10 days of culture, the perturbed tissues developed a cobbled or plaque-like morphology resembling scales rather than the feather-like filamentous structures that formed in unperturbed skin cultures. The results indicate that perturbation of CAM binding in tissues linked by one CAM can alter fates and interactions of cells linked by another, presumably by altering the amount or effect of inductive signals crossing the border between the inducing cell collectives. A computer model based on the notion that the response of L-CAM-linked epidermal cells to signals from N-CAM-linked dermal cells depends cooperatively on the degree of L-CAM linkage was found to generate hexagonal patterns for the unperturbed case and stripes after perturbation of L-CAM bonds.

The elaboration of morphological structures during embryonic development is dependent on inductive interactions between cell collectives having different histories (1). These interactions lead to differential gene expression, giving rise to new embryonic structures by altering the fates of cells in the participating tissues. Although inductive interactions have been defined in terms of the competence of cells at different times and places of interaction, the cellular and molecular genetic mechanisms underlying their changes in fate are not understood.

It has been proposed that the expression of cell adhesion molecules (CAMs) may provide an essential mechanochemical link between the genetic and epigenetic control of inductive events (2, 3). Studies on the localization of CAMs at inductive sites have shown that the two tissues involved express different CAMs on their cell surfaces according to definite modes or rules (4). CAM expression presumably mediates direct contact between cells in each of the adjoining tissues and establishes borders between such tissues as a result of differences in CAM binding specificities.

The induction of feathers is a particularly useful model for studying the causal role of CAMs because it results in a periodic pattern and it may be studied *in vitro*. In feather induction, signals of an unknown kind pass from epidermis to dermis and from dermis to epidermis, with different effects depending upon the stage of transaction and cellular competence. The experiments described in this paper were designed to study the effect of altering liver CAM (L-CAM)-mediated interactions among cells of the epidermal epithelium upon the induction of feathers by dermal condensations in the chicken skin. With the known effects of perturbation (for review, see ref. 3) of CAM binding on morphological integrity in mind, we constructed the following paradigm: (*i*) establish organ culture explants of the dorsal skin of the developing embryo; (*ii*) use antibodies to L-CAM to perturb intercellular linkages in the ectodermally derived epidermis by specifically blocking L-CAM-mediated binding; (*iii*) search for changes in mesodermal tissue linked by neural CAM (N-CAM), for changes in the hexagonal symmetry of feather germ pattern, and for long-term changes of morphology that ensue in culture. Our hypothesis was that disturbance of linkage by one CAM in a cell collective (epidermis) would alter the response of that collective to inductive signals or change the sequence or character of signal transfer to a second collective (mesoderm). Because the paradigm was designed specifically to avoid mechanical disruption in structures of the mesoderm (which are linked by N-CAM and not by L-CAM), ensuing changes in the pattern of that tissue would most likely reflect alterations in the coupling of CAM binding to signal response or signal transfer or both.

Application of anti-L-CAM antibodies to cultures for 3 days resulted in the formation of striped rather than circular mesodermal condensations and the underlying pattern of these stripes deviated from regular hexagonal symmetry. Instead of forming the normal filamentous structures characteristic of long-term (10 day) feather cultures *in vitro*, the perturbed cultures developed "cobblestone" or plaque-like structures. The data support the hypothesis that CAM interactions can influence the effects on patterning of inductive signals transferred across the borders of CAM-linked collectives.

MATERIALS AND METHODS

White Leghorn chicken eggs were purchased from a commercial hatchery and incubated at 37.5°C. The dorsal skin of embryos of 6½–7 days' incubation was peeled by blunt dissection and set dermal side down on a collagen-coated Nuclepore filter (8-μm pores) supported by a stainless steel organ culture grid (Falcon). The explant assembly was mounted in an organ culture dish (Falcon) and underlain with

Abbreviations: H&E, hematoxylin/eosin; L-CAM, liver cell adhesion molecule; N-CAM, neural cell adhesion molecule.

medium, either alone or containing an appropriate Fab' preparation. All cultures were grown and maintained at 37°C in 10% CO_2 balanced with air in medium NCTC 135 (GIBCO) with gentamycin added. Cultures grown for 3 days were fed daily with an appropriate mixture of medium and Fab'; cultures grown for 10 days were fed every 2 days. Fab' fragments of rabbit IgG were prepared as described (5). Nonimmune rabbit antibodies were prepared from serum from commercially raised rabbits (Dutchlands). Antibodies against whole liver cells were prepared as described (6). The anti-L-CAM used in these studies was a pool of antibodies from a number of bleedings of a single rabbit that was immunized with undenatured, affinity-purified Ftl fragment of L-CAM (6). Comparable results were obtained with a different preparation raised by using L-CAM denatured by $NaDodSO_4$ as an immunogen. The antibodies bound only to L-CAM polypeptides in immunoblots of liver and skin extracts (6, 7).

At the end of the culture period, explants were fixed overnight in Bouin's fixative. All samples were then decolorized in 70% ethanol saturated with Li_2CO_3 and stained overnight with borax carmine (8). After destaining with acid alcohol, the whole mounts were photographed by transmitted and reflected light using a Wild dissecting microscope. Samples were then processed for embedding in methacrylate (JB-4, Polyscience, Warrington, PA) according to the manufacturer's instructions or in paraffin (Tissueprep 2, Fisher) by standard methods (9). Transverse sections (2 μm) of cultures embedded in methacrylate were cut with a glass knife, mounted on slides, and stained with toluidine blue. Paraffin sections (10 μm) were cut *en face*, mounted on glass slides, and stained with hematoxylin/eosin (H&E). To assess mitotic activity, 3-day explants were incubated with medium containing 20 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per ml for 2 hr, embedded in paraffin, sectioned, and processed for autoradiography (10) using NTB-2 emulsion (Eastman). Total nuclei and labeled nuclei were counted in five to seven regions where the cells were clearly in a condensed or uncondensed state and the counts were normalized by area.

RESULTS

Feathers of the chicken develop in several different independent tracts (11). We chose to study the spinal tract because it covers a large area of the developing skin and because of its relative ease of manipulation. This tract develops visibly starting at 6–6½ days after laying (stage 29–30). The skin at this stage of development is a sheet of epithelium overlying a layer of loose mesenchyme. The appearance of a dense dermis formed from somitic mesodermal cells is followed by formation of epidermal placodes in the lumbar midline (12) in an anterior-posterior direction; rows of placodes then propagate mediolaterally yielding hexagonal arrays (12). New feather germs are formed in dorsal skin in a mediolateral direction at a rate of one row per 6 hr *in vivo* and one row per 15 hr *in vitro*, and an area is predetermined to form feathers several hours before any morphological change occurs laterad (13, 14). As a placode forms in the L-CAM-linked (7, 15, 16) epidermal sheet, the underlying mesenchyme forms N-CAM-positive cellular condensations underneath the placodal region (15). These condensations grow and expand, and as the overlying ectoderm loses its placodal thickening, the mesodermal masses expand further and form the protruding feather buds.

Uncultured whole mounts of the skin showed one to three rows of feather rudiments forming in the midline, with featureless tissue extending laterally (Fig. 1A). Skin explants grown in organ culture with medium alone or with medium containing Fab' fragments of nonimmune rabbit antibodies or antibodies raised against whole liver cells developed a
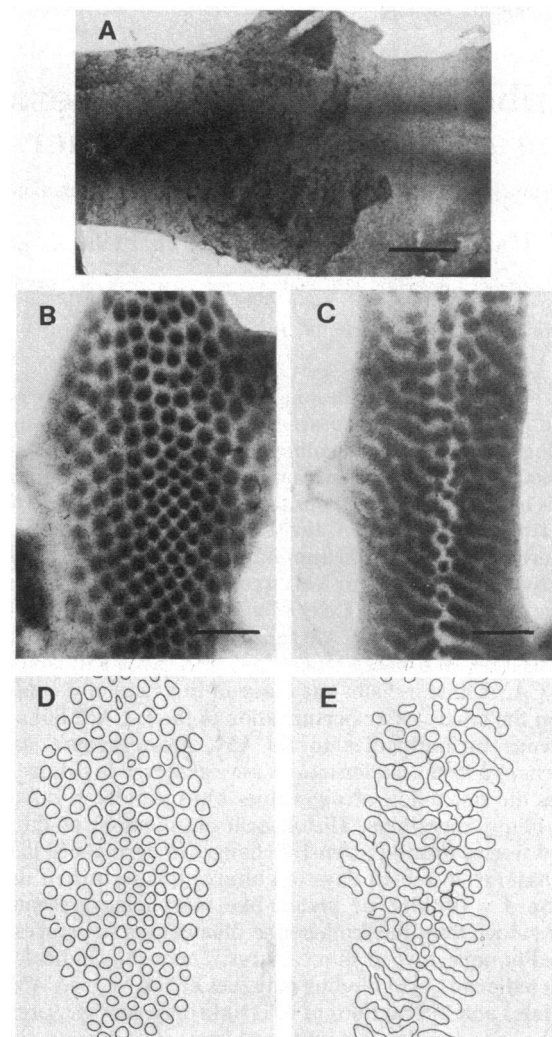

FIG. 1. Whole mounts of unperturbed and perturbed skin explants. (*A*) Uncultured skin removed from a 7-day embryo. (*B*) Seven-day embryo skin cultured for 3 days with 1 mg of nonimmune rabbit Fab' per ml. (*C*) Seven-day embryo skin cultured for 3 days with 1 mg of anti-L-CAM Fab' per ml. (*D* and *E*) Tracings of the patterns of condensations in *B* and *C*. (Scale bars = 1 mm.)

roughly hexagonal pattern of circular cell condensations (175–220 μm in diameter) after 3 days (Fig. 1 *B* and *D*). At the edges of the explants the staining of the condensations was lighter, they appeared to be more diffuse, and on occasion they were oval in shape.

Explants grown in the presence of Fab' fragments of antibodies to L-CAM showed a concentration-dependent disruption of the overall pattern of condensations. When cultured with 5 mg of Fab' per ml, the explants had few if any condensations and even the central condensations that had begun forming before the skins were dissected appeared small and distorted (not shown). Explants grown in the presence of 1 mg of Fab' per ml had a distinctive pattern (Fig. 1 *C* and *E*) that differed greatly from the unperturbed pattern: instead of being circular, the condensations formed stripes and the areas between the stripes were stained more darkly than the areas between unperturbed circular condensations. A second striking change was seen in the symmetry of the condensations. Whereas the circular condensations in the untreated skin formed in a roughly hexagonal pattern, the stripe condensations in the treated skin were occasionally formed in a diagonal direction coincident with one of the axes of the hexagonal pattern, but often extended in a directly mediolateral direction that was inconsistent with the sym-

metry of the unperturbed pattern. Adjacent stripes were usually parallel, giving an appearance of local order. The width of the stripes was roughly equal to the diameter of a normal circular condensation and most stripes had wavy borders, suggestive of possible fusion of developing centers. The stripes varied in length and occasionally fused or bifurcated. This was not observed in the midline of the explants, where part of the medial row of feather germs had already formed prior to the dissection, suggesting that the effects of anti-L-CAM were not degenerative but rather were directly on the development of the skin structures. Comparable results to those described above were obtained in at least 10 different experiments, with >40 explants, using the two different antibody preparations of proven specificity for L-CAM.

Histological Analysis. Transverse sections of explants of skin cultured for 3 days in medium alone or in the presence of nonimmune Fab' showed an epidermal layer that consisted of two epithelia (Fig. 2A). The periderm was a single-layered squamous epithelium on the top surface of the explant. The main epidermal epithelium was cuboidal in areas that were not developing feather germs and columnar or pseudostratified in the developing germs. In the dermal layer of the developing feather germ, the cells in each condensation formed a compact mass of closely apposed cells. Previous localization studies in embryos have demonstrated that the cells in these condensations express N-CAM as the induction proceeds (15, 16), beginning with a few cells and finally occupying most of the condensation. Sections that were cut through the dermis in the plane of the skin explant (Fig. 2 C and D) clearly showed the accumulation of dermal cells in the feather germs and the sparseness of the cells between the germs (100% more cells per unit volume in the condensations as shown by counts of nuclei; see Table 1). The condensations also displayed >8 times greater density of [^{3}H]thymidine-labeled nuclei at 3 days than the uncondensed regions (see Table 1); the labeling index was 4 times higher inside the dermal condensations. Cells in condensations also had much more compact morphologies than those outside the condensations, and part of the striking difference in H&E-stained sections was due to the darker staining of the cytoplasm in the cells making up each condensation.

Cultures grown in the presence of anti-L-CAM Fab' (1 mg/ml) for 3 days (Fig. 2B) showed a thicker periderm that appeared to be more loosely associated with the underlying epithelium. However, there was no gross distortion of the main epithelial layer and no gross mechanical disruption of the epidermal–dermal interface. Dermal cells in cultures perturbed with anti-L-CAM were not accumulated in the tight distinctive circular structures seen in the unperturbed cultures. Although there were a few areas with a sparse mesenchymal appearance, the majority of the dermal cells was present in a dense sheet with stripe-like regions of higher density. Sections in the plane of the skin (Fig. 2 E and F) showed this pattern clearly. Differences in cell density in the most dense and least dense regions were less extensive than in unperturbed cultures (only 20% more cells per unit volume in condensations; see Table 1). In striking contrast to the unperturbed case, the uncondensed dermis at 3 days contained a higher proportion of [^{3}H]thymidine-labeled cells than the condensed dermis ($\approx$2-fold more, see Table 1). Moreover, even cells in regions of lowest density showed compact morphology and darker staining with H&E, further diminishing the visible difference between condensed and uncondensed regions.

Long-Term Morphological Changes. To determine the effect of early antibody treatment on later morphogenesis and on the fate of the feather germs, skin explants were also grown in organ culture for 10 days. Explants grown in the absence of specific antibodies (Fig. 3A) developed rudimen-
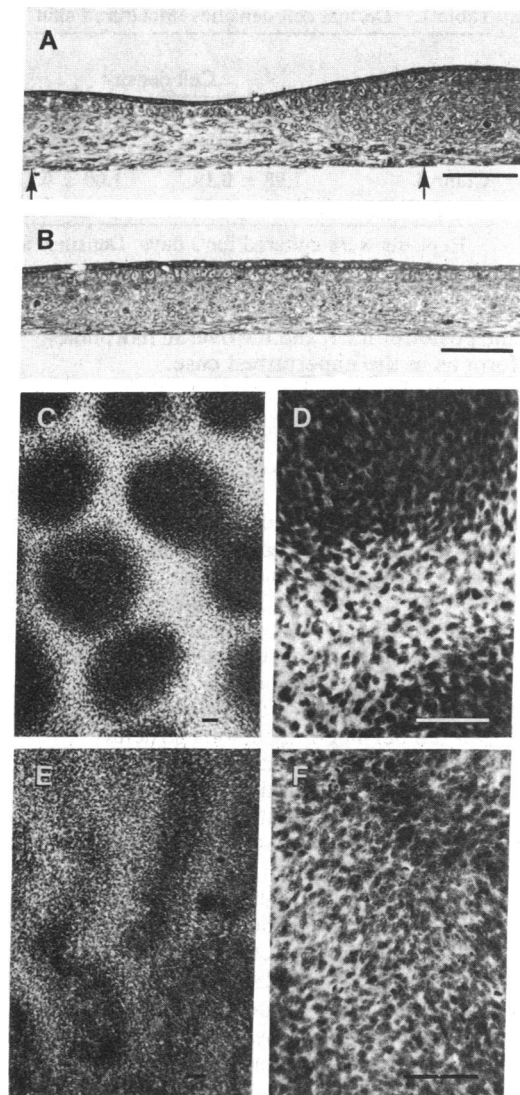

FIG. 2. Sections of skin explants cultured for 3 days without and with anti-L-CAM. (A and B) Transverse methacrylate sections (2 μm), stained with toluidine blue, of explants cultured with 1 mg of nonimmune Fab' per ml (A) or 1 mg of anti-L-CAM Fab' per ml (B). The arrows in A indicate the positions of dermal condensations covered by an epidermal placode. (C–F) Paraffin sections (10 μm), stained with H&E, of skin explants cultured for 3 days, cut in the plane of the skin to demonstrate the distribution of dermal cells. (C) Skin from 7-day embryo, cultured for 3 days in medium alone. (D) Higher magnification of an area in C. (E) Skin from 7-day embryo cultured for 3 days in the presence of 1 mg of anti-L-CAM Fab' per ml. (F) Higher magnification of an area in E; compare with D. (Scale bars = 50 μm.)

tary filamentous structures that were similar in appearance to feather filaments that develop on skin explants grown on chorioallantoic membrane cultures (17). These rudimentary structures are thick circular buds that are bent over in an anteriopposterior direction, most of which show a fine filamentous structure growing out of the posterior end.

Cultures that were maintained in the presence of antibodies to L-CAM for 2–3 days and then cultured with medium alone for the remainder of the 10-day culture period had a radically different morphology (Fig. 3B). The filamentous feather-like structures were replaced by knobby, cobbled structures, often fused in groups of two or three, that resembled scale rudiments forming on cultured foot skin (17). Only a few of these elements showed a thin filamentous structure growing

Table 1. Dermal cell densities in cultured skin

| Sample | Cell density | | | [^{3}H]Thymidine-labeled cell density | | |
	Dermal condensation	Uncondensed dermis	Ratio*	Dermal condensation	Uncondensed dermis	Ratio*
Control	1.98 ± 0.19	1.00 ± 0.11	2.0	0.32 ± 0.04	0.04 ± 0.03	8.4
Anti-L-CAM	1.69 ± 0.16	1.43 ± 0.10	1.2	0.17 ± 0.04	0.35 ± 0.05	0.5

Explants were cultured for 3 days. Densities are expressed as number of nuclei per 1000 μm^3.
*Condensed/uncondensed ratio.

out of the posterior face, and the overall morphology was not as uniform as in the unperturbed case.

DISCUSSION

The present experiments were designed to examine the causal role of a CAM in events leading to embryonic induction. Despite the fact that antibodies to L-CAM do not directly affect dermal cell adhesion, their addition to skin explants undergoing induction strongly altered the pattern of dermal condensations in the feather germs. Affected explants formed stripes instead of hexagonal arrays of circular condensations. Disruption caused early thus did not lead to complete disorder but rather to a new pattern that in long-term cultures gave rise to scale-like cobbled structures rather than feather-like filamentous structures. These coordinate alterations in early and late developing morphology suggest that normal definition of the pattern of feather primordia through signals that cause dermal cells to condense and proliferate must depend upon direct cell–cell adhesion in the epidermal sheet.

Because the observed changes were so widespread, the cultures and antibody preparations were carefully monitored to rule out adventitious effects. There was no evidence that the highly specific antibodies to L-CAM used in these experiments were toxic for cells nor was their effect purely mechanical: the cultured tissues did not collapse, wrinkle, or shear at any dose of <5 mg/ml. Condensations that had formed prior to perturbation remained unaltered, indicating that the effect of the antibody treatment was not degenera-
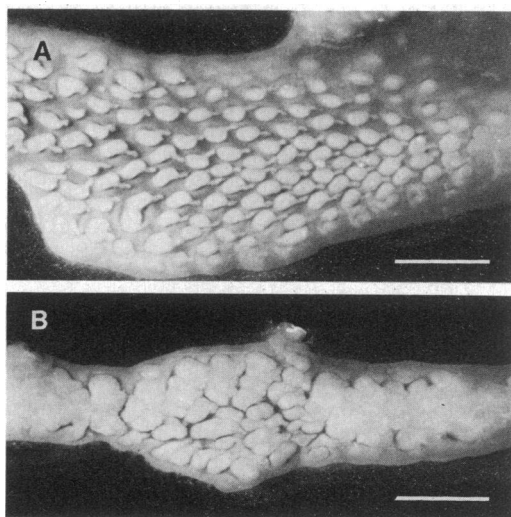

FIG. 3. Whole mounts of unperturbed and perturbed skin explants grown in culture for 10 days. (A) Skin from 7-day embryo cultured for 2 days in the presence of 1 mg of nonimmune Fab' per ml and then for 8 days with medium alone. (B) Skin from 7-day embryo cultured for 2 days in the presence of 1 mg of anti-L-CAM Fab' per ml and then for 8 days with medium alone. (Scale bars = 1 mm.)

tive. Although antibodies at a dose of 5 mg/ml completely disrupted epidermal cell–cell adhesion, at all lower doses, removal of the antibodies from culture allowed cells to readhere and reform epithelial structures, indicating that the direct effects of the antibodies were reversible. Functional tests (W.J.G. and G.M.E., unpublished) of the antibodies confirmed their specificity as revealed by immunoblots: anti-L-CAM inhibited aggregation of epidermal cells *in vitro* but had no effect on dermal cells; anti-N-CAM partially inhibited dermal cell aggregation but had no effect on epidermal cells.

The fact that the pattern of accumulation of N-CAM-linked dermal cells changes as a result of direct perturbation only of L-CAM-linked epidermis indicates that a signal sent down from the epidermis to the dermis or an epidermal response to a signal sent up from the dermis (or both) is dependent on the integrity of the epidermal cell collective linked by L-CAM. In view of their different locations and different binding specificities, it seems unlikely that L-CAM or N-CAM itself is directly involved in signaling between epidermis and dermis.

These conclusions have been incorporated into a computer model (L.H.F. and G.M.E., unpublished) of feather germ induction that generates the normal and the antibody-perturbed patterns. The model differs from previous proposals (18–21) in that it explicitly specifies a causal role for CAMs in the response of cell collectives to inductive signals. In the model, the lateral advance of the dense dermis (in this instance, a result of mitotic activity) is a major constraint on the order of formation of actual condensations (21). Experimental evidence (reviewed in ref. 21) suggests a minimum of two inductive signals (Fig. 4A): E_s, produced by L-CAM-linked epidermal cells, and D_s, produced by N-CAM-linked dermal cells. We treat the signals as diffusible morphogens, but the model is also consistent with direct cell–cell signaling. E_s acts on mesenchymal cells within dense dermis to increase their mitotic rate, turn on N-CAM production, and form condensations. Dermal cells in condensations then produce D_s, which acts upon epidermal cells to induce placode formation and to down-regulate production of E_s over an area slightly larger than each condensation. This eventually halts the growth of the underlying dermal condensate. In normal epidermis, down-regulation of E_s occurs in a cooperative manner, reflecting cell–cell interactions mediated by L-CAM (solid curve in Fig. 4A). Anti-L-CAM antibodies act exclusively on epidermis and could alter production of E_s in three ways: (*i*) by changing the effect of D_s in down-regulating E_s production, (*ii*) by changing the baseline rate of production of E_s itself, or (*iii*) by changing the time constants of epidermal cellular response to D_s. When tested in computer simulations, increasing the net production of E_s by combinations of the first two possibilities led to stripes in a robust manner; the third possibility also led to stripes but only in a narrow parameter range. We therefore assumed in further modeling experiments that disrupting L-CAM linkages of epidermal cells leads to increased production and decreased down-regulation of E_s in epidermis by interfering with intercellular cooperativity (dashed curve in Fig. 4A).
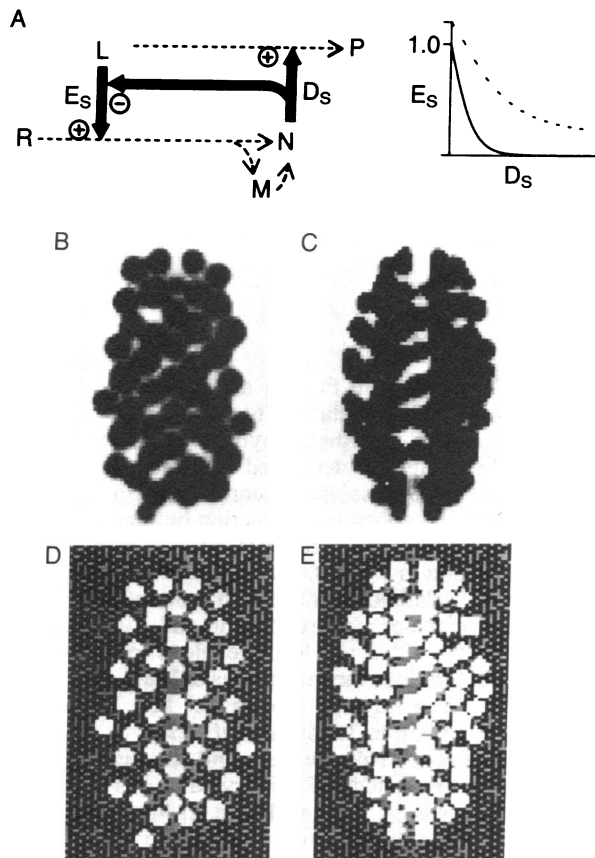
FIG. 4. Computer model of feather pattern formation. (*A*) Epidermal cells (L) linked by L-CAM produce a signal (E$_s$). Dermal cells in mesenchyme (R) respond to E$_s$ by increased mitosis (M), by production of N-CAM (N), and by production of another signal (D$_s$). D$_s$ induces placode formation (P) and down-regulates epidermal production of E$_s$. Cellular responses to the inductive signals are stochastic. Graph at right shows plots of the production of E$_s$ as a function of the level of D$_s$. In the normal case, the interaction is highly cooperative (solid curve); in the antibody-perturbed case, the curve shows a higher baseline and less down-regulation of E$_s$ (dashed curve), due to decreased cooperativity between epidermal cells. The simulations involved 9600 epidermal and 3850 dermal cells, each of which underwent iterated cycles of signaling and response according to the scheme in *A*. Patterns in *B* (epidermal E$_s$ level) and *D* (dermal condensations) correspond to normal development after 350 cycles; those in *C* and *E* correspond accordingly to perturbed development in which there was decreased epidermal cooperativity. In *B* and *C*, darker regions represent areas of greater down-regulation of E$_s$ due to higher levels of D$_s$. In *D* and *E*, black areas contain no cells, grey areas are mesenchymal cells, and white areas are cell condensations, expressing N-CAM and D$_s$. Compare these panels to *D* and *E* in Fig. 1.

Unperturbed development is simulated in Fig. 4 *B* and *D*. The first condensations form sequentially from the center outward in the midline row and the final hexagonal pattern depends upon the initial formation of this row. As the front of dense dermis moves laterally, new condensations form lateral to the *spaces* between condensations in the adjacent medial row. The reason is that as the dense dermis migrates laterally, these locations are the first locations encountered that have sufficiently high values of E$_s$ to form a condensation.

Simulation of antibody-perturbed development (Fig. 4 *C* and *E*) also began with a normally developed midline row of condensations. By decreasing the cooperativity of the function reflecting down-regulation of E$_s$ (see Fig. 4*A*), striped

patterns resembling the experimental patterns were obtained. In the simulation, anti-L-CAM acted on the epidermis to prevent E$_s$ from being sufficiently down-regulated and, as a result, dermal cell condensation continued laterally, forming stripes of cell condensations. Between condensations, however, E$_s$ was still sufficiently down-regulated to prevent condensation, thus ensuring stripe formation. Further tests and elaborations of this model await perturbation analysis using anti-N-CAM as well as anti-L-CAM.

The present study provides a direct demonstration that a CAM can be causally involved in the complex chain of embryonic induction. The results fulfill one of the main predictions of the regulator hypothesis relating the mechanochemical functions of CAMs to inductive signaling (2, 3). Another prediction of this hypothesis is that changes in bordering cell collectives mediated by CAM expression may also affect the subsequent or late expression of specific historegulatory genes at various induction sites. In the case of feather induction, studies of the coordinate spatiotemporal expression of CAM genes and keratin genes may provide a particularly critical test of this prediction. L-CAM is obviously not the only gene product that might act as a mechanochemical mediator in the complex inductive processes of feather histogenesis. Perturbation of dermis by anti-N-CAM and disruption of cell–substrate interactions mediated by other molecules may reveal additional pathways connecting genetic regulation to epigenetic events in development.

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