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Morphoregulatory Molecules†

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Almost no other biochemical event bridges so many levels of biological organization as cell adhesion. Contrary to the notions that adhesion is a simple "gluing" event mediated by charge or noncovalent forces (Curtis, 1967) or even a form of individual cell recognition acting through cell addressing (Sperry, 1963), adhesion is a spatially and temporally dynamic process mediated in a series of stages by at least three different families of molecules [see Edelman and Thiery (1985)]. It is under cellular control as a function of place, affecting gene expression at one end of the spatial scale and morphogenesis at the other. Adhesion was an essential mediating event in the evolution of metazoan life, and in present day complex animals, it plays a central role in development and metamorphosis, as well as in phenomena such as bone repair and regeneration.

The developmental importance of adhesion has been particularly well appreciated since the pioneering studies of Holftreter (1948a,b) on reassociation in vitro of embryonic tissues after dissociation. But because of the wide-ranging effects and multiple contexts of cell adhesion, it has been difficult until recently to relate its biochemical bases to the analysis of morphogenetic events. Three developments have served to open this area to structural and functional analysis at the molecular level. The first was the appreciation that adhesion events, while certainly involving molecular specificity of binding, were also regulatory events involving various forms of cell surface modulation that result in altered patterns of synthesis and binding (Edelman, 1976, 1983). The second was the elaboration of molecular means to analyze the genetic control of morphogenesis, particularly studies carried out in *Drosophila* [see Scott and O'Farrell (1986)]. The third was the development of biochemical assays and analyses for adhesion molecules—CAMs or cell adhesion molecules (Brackenbury et al., 1977; Thiery et al., 1977), SAMs or substrate adhesion molecules and their receptors (Yamada, 1983; Hynes, 1987; Ruoslahti & Pierschbacher, 1987), and CJMs or cell junctional molecules [see Stoker (1987) and Edelman and Thiery (1985)]. A synthesis of the ideas emerging from these developments suggests that very special mechanisms of cellular signaling evolved to relate the expression of genes that specify these molecules with the expression of genes that specify other developmentally important proteins, particularly those concerned with histodifferentiation. The resultant mechanochemical and regulatory effects of expressed CAMs and SAMs upon cell movement and cell division play a central role in morphogenesis. CAMs, SAMs, and CJMs are morphoregulatory molecules—molecules whose gene expression affects cell patterning and tissue signaling through mechanochemical effects on cell linkage, shape, and movement.

In this paper, I want to provide some examples of how coordinate regulation of cell–cell and cell–substrate interactions is ultimately correlated with morphological pattern, first, by cycles of expression of CAMs to form borders, and second, by the action of elaborate multimolecular binding networks involving SAMs in such a way as to alter the patterns of cellular primary processes. My emphasis shall be on pattern-forming events, not simply on differentiation or gene expression. I shall deliberately pick only a few examples and shall not attempt an exhaustive review [for further references, see Edelman and Thiery (1985), Bock and Clark (1987), and Edelman (1986b,c)].

CAM Structure and Gene Organization. At least six different CAMs have now been identified (Edelman, 1986a–c). Of these, the most thoroughly studied are the two primary CAMs, N-CAM (neural cell adhesion molecule, the first to be isolated by strict criteria) and L-CAM (liver cell adhesion molecule). They are named primary CAMs because they appear early in development in tissues of all germ layers but they are also used in later histogenesis. An example of a secondary CAM, which appears somewhat later in development, mainly in neural tissues, is Ng-CAM [neuron–glia cell adhesion molecule (Grumet et al., 1984a,b)].

All of these CAMs are large intrinsic cell surface glycoproteins that are mobile in the plane of the membrane (Gall

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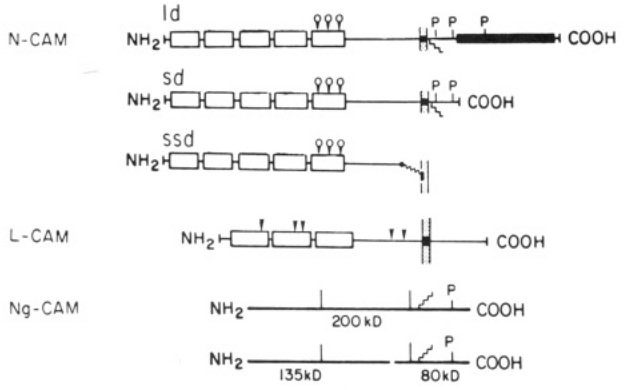

FIGURE 1: Diagrams of the linear chain structure of two primary CAMs (N-CAM and L-CAM) and of the secondary Ng-CAM. N-CAM is comprised of three chains that differ in the size of their cytoplasmic domains and the mode of attachment to the cell surface. The cell membrane is represented by a stippled area within vertical bars. Three main polypeptides are generated by alternative mRNA splicing from RNA transcribed from a single N-CAM gene. The ld (large domain) polypeptide contains 261 more amino acid residues (black bar) in ths region than does the sd (small domain) polypeptide (thin bar). Zigzag lines refer to sites of palmitate attachment. A third, and the smallest (ssd), polypeptide is attached to the membrane by phosphatidylinositol (to which the extracellular zigzag line refers in this case only). Most of the carbohydrate is covalently attached in the fifth homology domain at three sites (O). Attached to these carbohydrates is polysialic acid. L-CAM has three homology regions and four attachment sites for carbohydrate (arrowheads show five potential sites) but lacks polysialic acid. Its structure is completely different from that of N-CAM. Ng-CAM is shown as a major 200-kDa chain. There are two components (135 and 80 kDa) that are probably derived from a posttranslationally cleaved precursor. Each is related to the major 200-kDa chain (which may be this precursor), and the smaller is arranged as shown on the basis of a known phosphorylation site.

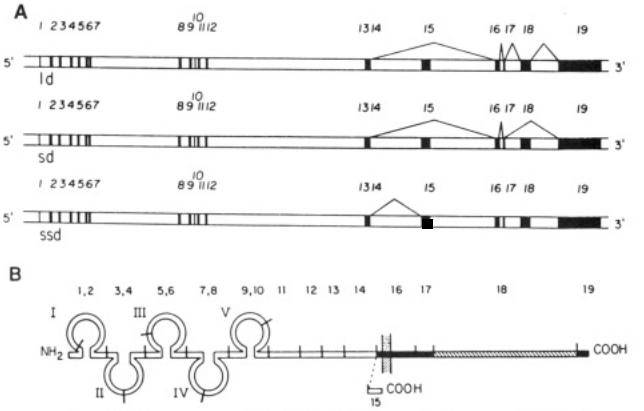

FIGURE 2: (A) Structure of the N-CAM gene in terms of the introns and exons that code for N-CAM mRNAs from the amino terminus of the protein through the 3′ end. The segments of the proteins specified by each exon are indicated on the model of N-CAM (B). Exons, 16, 17, and 19 specify regions shared by the ld and sd chains including the 3′ untranslated regions of the mRNAs. Exons 15 and 18 specify regions unique to the ssd and ld chain, respectively. (B) Model of N-CAM showing homologous loops (I–V) in the region common to all three polypeptides (open bar). The base of each loop corresponds to the proposed intradomain disulfide bond. The cell membrane is indicated by stippling between vertical bars, and the extracellular and intracellular regions are to the left and right, respectively. The membrane-spanning region and carboxyl-terminal segment common to the ld and sd chains are indicated by the solid bar, while the cytoplasmic domain unique to the ld chain is indicated by the hatched bar. The unique segment of the ssd chain is indicated by the open bar and dashed line below. Numbers 1–19 correspond to the exons in the chicken N-CAM gene, and their relative boundaries in the protein are noted by transverse lines.

& Edelman, 1981; Pollerberg et al., 1986). They each have distinctly different structures (see Figure 1), although there are multiple related forms of N-CAM and several other CAMs exist that are structurally (and, in all likelihood, evolutionarily) related to L-CAM (Hatta et al., 1985; Nose & Takeichi, 1986; Volk & Geiger, 1986). The complete primary structures of N-CAM and L-CAM have recently been deduced from sequence analysis of appropriate cDNAs in the chicken (Cunningham et al., 1987; Gallin et al., 1987) and subsequently for these molecules in the mouse (Barthels et al., 1987; Ringwald et al., 1988). In both cases, there is a high degree of homology for the corresponding CAMs, and certain CAM functions have obviously been evolutionarily conserved [see Hoffman et al. (1984) and Hall and Rutishauser (1985)].

Several major conclusions have emerged from all of these findings. One of the most striking is that the primary CAMs, N-CAM and L-CAM, are completely different in structure and appear to be evolutionarily unrelated. N-CAM is expressed as a number of major polypeptide chains (Figure 1), each having five successive homology regions, the first of which begins close to the N-terminus (Cunningham et al., 1987; Cunningham, 1988): the ld and sd polypeptides, which have different cytoplasmic domains; the ssd (small surface domain) polypeptide, which has no cytoplasmic domain but is linked to the cell surface by a phosphatidylinositol intermediate (Nybroe et al., 1985; Hemperly et al., 1986b; He et al., 1986); and a muscle-specific form, which has a 37 amino acid unique sequence in the extracellular portion beyond the fifth homology region and otherwise resembling the ssd chain (Dickson et al., 1987). The unique short extracellular sequence beyond the fifth domain of this form is rich in proline, serine, and threonine. The five homologous extracellular domains of N-CAM are identical in ld, sd, and ssd chain forms; each domain is linked by a single intrachain disulfide bond, and as we shall discuss later, each domain is homologous to the immunoglobulin superfamily (Hemperly et al., 1986b; Cunningham et al., 1987).

It has been shown that N-CAM is specified by a single gene at the *n-cam* locus on chromosome 9 in the chicken (D'Eustachio et al., 1985) and on chromosone 11 in the human (Nguyen et al., 1985). The structurally different major polypeptides arise in development at different times and places by alternative splicing of mRNA (Murray et al., 1986a,b). As shown by a detailed analysis of the chicken N-CAM gene (Owens et al., 1987) which spans over 80 kb in length, the three chains arise by splicing together nucleotide sequences corresponding to different combinations of exons (Figure 2). In particular cases, this event can be tissue specific. For example, the ld chain arises only in the nervous system, and another tissue-specific variant is seen in tissues such as skeletal and heart muscle (Murray et al., 1986b; Dickson et al., 1987). Such splicing events would be expected to alter the position, mobility, and cytoskeletal interaction of a given CAM chain, and they may also alter the effects of CAM interactions on signaling in the cell cortex.

Alternative RNA splicing is only one of several mechanisms of cell surface modulation known for CAMs; others (Edelman, 1986b,c) include changes in surface density (prevalence modulation), distribution on the cell surface (polarity modulation), and carbohydrate structure (chemical modulation). All of these forms of cell surface modulation would be expected to change the amount of cell binding or cell responses to local signals by changing the rate or strength of binding, the cell spacing between bound cells, or the cytoskeletal dynamics affecting signal processing and cell shape.

Attached to the fifth domain (Figure 1) at three sites in chicken N-CAM (Crossin et al., 1984) are oligosaccharides

containing a carbohydrate that is unusual in vertebrates, α-2,8-polysialic acid (Rothbard et al., 1982; Finne et al., 1983). In the so-called embryonic (E) form of N-CAM there is about 30 g of this carbohydrate per 100 g of polypeptide, and in the adult (A) forms there is about one-third as much. The names refer to the statisical predominance of these forms at different ages: although molecules having low amounts of the carbohydrate can be seen in early embryos and molecules with high amounts can be seen in certain locations in adult nerve, skin, and muscle, these forms are in the minority. As we discuss below, polysialic acid does not participate directly in N-CAM binding, but the presence of higher amounts of the carbohydrate does diminish binding rates (Hoffman & Edelman, 1983). One would therefore expect to see the A forms in regions where stabilization of the dynamics of adhesion is required as, for example, in the adult brain; in regions of constant regeneration such as the adult olfactory bulb, one sees the E form (Chuong & Edelman, 1984).

L-CAM (Figure 1), while unrelated to N-CAM in sequence, also has three and possibly four regions of internal homology of about 110 amino acids each (Gallin et al., 1987; Nagafuchi et al., 1987; Ringwald et al., 1988). At least two other molecules, N-cadherin or A-CAM (Hatta et al., 1985; Volk & Geiger, 1986) and P-cadherin (Nose & Takeichi, 1986), have been found to be structurally related to L-CAM. Aside from these molecules, which apparently constitute an L-CAM family, no homology to other proteins has yet been detected. Unlike N-CAM, there appear to be no disulfide bonds in L-CAM homology regions, and although oligosaccharides are attached at four sites, no unusual carbohydrates have been found. A most striking property of L-CAM is its dependence upon Ca^{2+} for both its conformation and binding; in the absence of this ion, the molecule is rapidly cleaved by proteases.

N-CAM and L-CAM are primary CAMs found very early in development on cells of all three germ layers. Ng-CAM (neuron–glia CAM) appears as a secondary CAM on postmitotic cells of the developing nervous system (Grumet et al., 1984a,b). It mediates binding of neurons to neurons and of neurons to astrocytes in in vitro tests (Grumet & Edelman, 1988), and it is strongly involved in fasciculation (side-to-side bundling) of neurites. It has been shown to be identical with the NILE glycoprotein (Friedlander et al., 1986), and it appears as a 200-kDa polypeptide, which is cleaved to 135- and 80-kDa chains (Figure 1). Although Ng-CAM is unrelated to N-CAM in its detailed structure, certain subfractions of Ng-CAM and N-CAM display the carbohydrate epitope HNK-1 (Abo & Balch, 1981; Chou et al., 1985; Tucker et al., 1984; Noronka et al., 1986; Kruse et al., 1984) bearing sulfated glucuronyl residues; this epitope is also seen in lymphocytes (Abo & Balch, 1981) and has been found on certain SAMs such as cytotactin and cytotactin binding proteoglycan (Hoffman & Edelman, 1987). Its functional role remains unknown.

Binding Properties and Mechanisms. Although thermodynamic data have been hard to come by because of the complexity and scarcity of the CAMs and their dependence on membrane attachment for full function, a number of mechanisms and binding dependencies have been analyzed by special kinetic assays or by use of transfection techniques applied to cells lacking a given CAM. As a result of these analyses, it has been determined that N-CAM, L-CAM, and Ng-CAM binding mechanisms are all specific and independent of each other. N-CAM and Ng-CAM binding mechanisms are Ca^{2+} independent, whereas that of the L-CAM family is Ca^{2+} dependent. In all three cases, it has been shown that

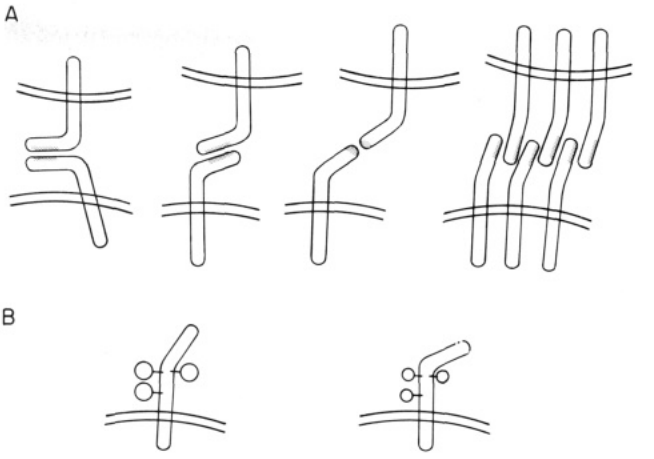

FIGURE 3: (A) Several hypothetical modes of N-CAM trans homophilic binding. From the left: N-CAM molecules on apposing cells may interact with each other in a manner similar to that of the pairing of domains in Ig molecules. In this mode, interacting chains would have the same N-to-C orientation, and the binding region of one molecule (shaded) would contact the equivalent region of the bound molecule. If the binding is not strictly analogous to that of Ig domains, the interacting chains may have opposing senses, and the binding region could be more or less extensive. If the binding region (shaded) is spatially distinct from its receptor on another molecule (unshaded), oligomeric or polymeric assemblies could be formed between adherent cells. All of these modes of binding would be facilitated by a flexible hinge region, with the possible exception of the end-to-end mode. (B) The large polysialic acid groups on embryonic N-CAM (left) may interfere with cis intermolecular interactions or with the proposed flexible hinge region. The reduced amount of carbohydrate on adult N-CAM (right) is consistent with less restricted intermolecular interactions and greater molecular flexibility.

binding is homophilic [CAM on one cell to the same CAM on the apposing cell (see Figure 3)]. This conclusion has been reached by performing assays on N-CAM and Ng-CAM linked to covaspheres or in artificial lipid vesicles (Hoffman & Edelman, 1983; Grumet & Edelman, 1988) and, for N-CAM and L-CAM, by transfection with appropriate cDNAs of mouse L cells that lack these CAMs (Edelman et al., 1987; Nagafuchi et al., 1987). In addition to homophilic binding between neurons, Ng-CAM binds neurons to astrocytes by a second mechanism, which is presumably heterophilic since astrocytes lack Ng-CAM (Grumet et al., 1984a,b; Grumet & Edelman, 1988).

The physicochemical assays (Hoffman & Edelman, 1983; Grumet & Edelman, 1988) that depend upon measurements of rates of aggregation for both N-CAM and Ng-CAM indicate that a 2-fold increase in surface density can result in a greater than 30-fold increase in binding rates. In changing the linkage of cells bound in a collective by a given CAM or in forming borders between two such collectives each linked by a CAM of different specificity, such nonlinear changes occurring by cell surface modulation could lead to rapid changes in cell interactions in vivo. This is also the case for chemical modulation: although α-2,8-polysialic acid is not directly involved in binding, changes in the amount of this negatively charged sugar (chemical modulation), probably as a result of turnover of E forms and replacement by A forms (Friedlander et al., 1985), can result in 3–4-fold changes in binding rates (Hoffman & Edelman, 1983).

As shown in Figure 3, in the case of N-CAM such changes may be the result of local changes in the conformation of the molecule as a hinged structure. The earliest (Edelman et al., 1983b) and most recent (Hall & Rutishauser, 1987) electron microscopic analyses are consistent with such a structure having N-terminal binding domains followed by a hinge. If

the analysis of the polarity of the molecule is correct, then the presence of multiple oriented N-CAM chains in higher order structures observed in the electron micrograph may imply the presence of alternating attachment sites (Figure 3) rather than of closed homophilic pairs. Whatever the case, hinged structures may facilitate trans homophilic binding in the presence of cell shape changes that would otherwise sterically hinder homophilic binding. The function of α-2,8-polysialic acid inserted on N-CAM at the three known sites of the fifth domain may be multiple: to affect cis spacing of molecules on the same cell, to alter the freedom of the hinge thus affecting apposition of terminal binding domains (Figure 3), and to alter cell-to-cell spacing by excluded volume and charge effects. As mentioned before, E forms may predominate when adhesion is being established (such as in formation of new neural connections), and A forms may predominate when stabilization is required (as in the adult brain).

It is important to understand that the occurrence of cell surface modulation indicates that the binding properties of homophilic CAMs are under direct and immediate control of the cells that they ligate. This is in direct contrast to certain SAMs, which, as extracellular molecules, bind to independent receptors on adhering cells, thus rendering control and multimolecular interactions more complex and indirect in their effects. Nonetheless, CAMs may also have additional subtleties in their binding: as noted, recent studies suggest that Ng-CAM, which is synthesized by neurons and not by glia (Grumet & Edelman, 1988), is homophilic in neuron–neuron binding and heterophilic in neuron–glia binding; moreover, N-CAM has been found to bind heparin sulfate in the neighborhood of its first two domains (Cole & Glaser, 1986; Cole et al., 1986).

Transfection by CAM cDNAs Leading to Acquisition of Specific Cell Binding. One of the most direct means of demonstrating that CAMs ligate the cells that synthesize them is to show that cells which ordinarily do not make a given CAM will aggregate specifically after being transfected with a cDNA for that CAM. This has been done recently for N-CAM (Edelman et al., 1987) and L-CAM (Edelman et al., 1987; Nagafuchi et al., 1987) with striking and clearly interpretable results (Figure 4). Mouse L-cells, which do not ordinarily express either N-CAM or L-CAM, were transfected separately with cDNAs for each of the N-CAM chains and for chick L-CAM via a chimeric cDNA (Edelman et al., 1987), and clonal lines were obtained. In each case, the appropriate CAM was synthesized. For the sd and ld chains, there was expression at the cell surface, but for the ssd chain, there was only cytoplasmic synthesis or secretion. This suggests that the mechanism for phosphatidylinositol linkage was not present or not activated in these cells; control experiments using COS cells transiently transfected with cDNA for ssd chains indicate that such a mechanism could be activated, yielding ssd chains linked by phosphatidylinositol at the cell surface (B. Murray, J. Hemperly, and G. M. Edelman, unpublished results).

Binding assays on transfected cells showed that sd and ld chains of N-CAM and L-CAM each at their respective cell surfaces were capable of linking the cells in aggregates and, in the case of N-CAM expression, of binding neuron vesicles containing N-CAM. In all cases, binding was inhibited by appropriately specific antibody fragments. L-CAM aggregation was particularly striking and provided strong evidence for a homophilic mechanism of binding inasmuch as untransfected L-cells would not bind with transfected cells expressing L-CAM.

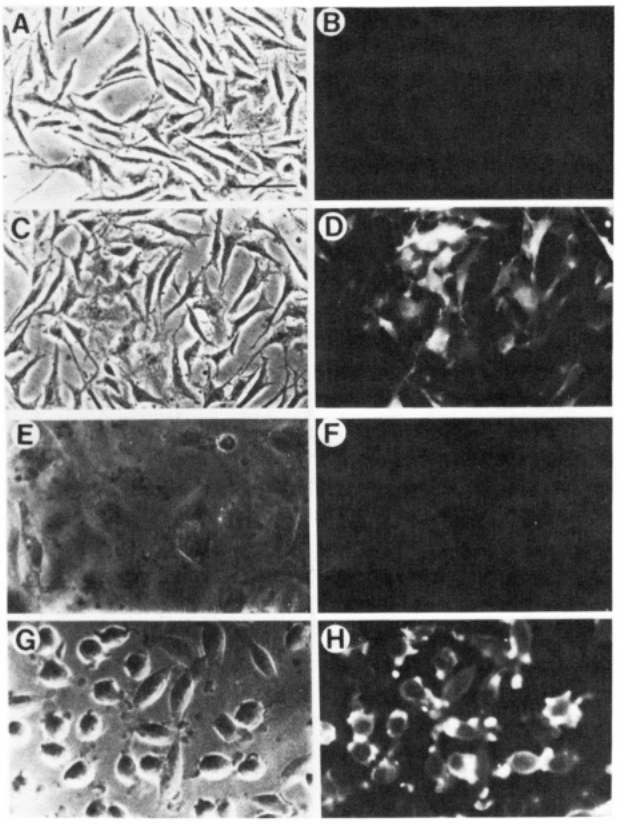

FIGURE 4: Matched phase-contrast (A and C) and fluorescence (B and D) photographs of mouse L cells transfected with L-CAM cDNAs; the cells yielded no immunofluorescent staining for N-CAM (B) but gave bright uniform surface staining for L-CAM (D). (E–H) Cell surface expression of N-CAM in transfected cells: matched phase-contrast (E and G) and fluorescence micrographs (F and H) of a cell line transfected with cDNA for the ssd chain of N-CAM (E and F) and of a cell line transfected with cDNA for the ld chain of N-CAM (G and H) immunofluorescently stained with rabbit antibodies to chicken N-CAM. The ssd transfectant had no detectable cell surface immunoreactive material (F), although it secreted the molecule into the medium as detected by electrophoresis and immunoblotting. The ld transfectant was brightly stained at the cell surface and also in blebs (H); this clone, like others that express N-CAM at the surface, had a rounded morphology with membrane blebs and adhered less tightly to the culture dish than did untransfected cells.

Phenotypic changes in shape were seen in cells that possessed the N-CAM ld and sd chains on their surfaces but not for those expressing ssd chains and releasing them into the medium (Edelman et al., 1987). Whether these changes can be attributed to primary association of carboxyl-terminal CAM domains with the cell cortex or to cis binding effects resulting from the lack of α-2,8-polysialic acid remains to be determined. The transfection paradigm provides a powerful means of assessing such variables: hybrid cDNAs specifying the carboxyl-terminal domains of one CAM and the extracellular binding domains of another can be used. Alternatively, cells can be transfected with more than one CAM cDNA to test for the effects of expression of multiple CAMs of different specificity on border formation. The relation of such border formation to the regulation of CAM expression in vivo is an important subject to which we now turn.

Regulation of CAM Expression in Vivo. The most striking aspect of the expression of different CAMs during development, metamorphosis, and regeneration is its site-dependent regularity in different individuals of the same species. It is clear that CAM expression is controlled epigenetically and that it is different in different cellular contexts, reflecting different tissue interactions and developmental stages. This has been

shown by examining the sites and stages of appearance of each specific CAM at different times of development from the fertilized egg onward by immunohistochemical methods (Crossin et al., 1985; Chuong & Edelman, 1984; Daniloff et al., 1986a,b; Levi et al., 1987). In addition, perturbation experiments have shown that antibody blockade of CAM binding results in altered morphology and that mechanical disruption of morphology can in some cases lead to changes in CAM expression. As a result of such explorations, it has become apparent that, during development, primary CAMs (and possibly the secondary CAMs) follow rules according to which they are expressed or down-regulated at the cell surface during border formation between tissues (Edelman, 1986a–c).

To appreciate the significance of these rules, it is important to realize that they are correlated with certain primary processes of development including cell division, movement, and death, all of which act as major driving forces leading to cell patterns. The binding via CAMs (and SAMs) can alter the sequence and effects of such driving forces, resulting either in the formation of cell collectives or in their transformation into groups of migrating cells. The most specific such transformation is from epithelia (sheets of cells linked by CAMs, SAMs, and CJMs in a polar fashion from the apex to the base of their constituent cells) to mesenchyme (collections of loosely associated cells interacting with SAMs of the extracellular matrix and generally having down-regulated CAMs at their surface). Separate epithelia and mesenchymes can interact with each other or with themselves, leading to embryonic induction, a complex process of milieu-dependent differentiation (Gurdon, 1987) leading to expression of genes for the morphoregulatory molecules (CAMs, SAMs, and CJMs) as well as of those for tissue- and cell-specific molecules.

Both of the primary CAMs considered here are expressed on all cells at early stages (e.g., blastoderm or blastula) of chick and frog embryos (Crossin et al., 1985; Edelman, 1986a–c; Levi et al., 1987). After cell movement and gastrulation take place, there occurs a remarkable segregation of CAM expression: N-CAM is seen on cells of the neural plate and reappears on condensing mesoderm whereas L-CAM is seen alone or appears together with N-CAM in epithelia. Upon formation of mesenchyme, N-CAM is down-regulated (often during cell movement) only to be reexpressed when mesenchymal cells condense to form epithelia (N → O → N, rule I). Often, on early epithelia, one observes both N-CAM and L-CAM; subsequently, one or the other is lost (NL → L or NL → N, rule II). At many sites where mesenchyme acts to induce epithelia, a collective of cells obeying rule I adjoins a collective of cells obeying rule II. We shall discuss a striking example, the feather, later. Often one may observe in the same developing tissue (e.g., the kidney or the feather) multiple cycles of primary CAM expression in which CAMs are repeatedly up-regulated and down-regulated in this fashion at different stages of histogenesis.

Space does not allow more than a brief listing of a few examples of CAM expression sequences; the interested reader may consult other reviews (Edelman, 1985, 1986b,c) for details and further bibliography. Some salient examples in four different tissues are as follows. (1) Nervous system: (a) formation of the neural plate and tube as already mentioned; NL → N; (b) migration of neural crest cells, N → O → N (Edelman et al., 1983a); (c) neural histogenesis including neurite formation and fasciculation—expression of Ng-CAM along with N-CAM on the same cells and processes (Thiery et al., 1985a,b; Daniloff et al., 1986a,b). (2) Muscle: (a) early development—myotubes show N-CAM on entire surface but lose it except on the motor endplate and basement membrane after functional synapses mature (Rieger et al., 1985; Covault & Sanes, 1985); (b) regeneration—after cutting a nerve, N-CAM reappears on the myofibril surface, down-regulating only after regeneration is well advanced (Daniloff et al., 1986b). (3) Kidney: L-CAM is seen on Wolffian ducts (inductor tissue) and after induction N-CAM appears on mesonephric mesenchyme; after induction, L-CAM appears on collecting tubules (Thiery et al., 1984). (4) Gut: N- and L-CAM are seen in epithelia and N-CAM then disappears leaving L-CAM (Crossin et al., 1985). Such examples could obviously be multiplied. We shall discuss three additional ones later when we consider the coordinate expression of CAMs and SAMs during morphogenesis.

How do CAMs function in relation to morphogenesis? It has been shown that blockade of CAM binding both in vitro and in vivo leads to alterations of morphology. Examples include interference with side-to-side interaction of axonal fibers of neurons by Fab′ fragments to Ng-CAM (Hoffman et al., 1986; Schachner et al., 1985), perturbation of layer formation in the retina in organ culture by Fab′ fragments of antibodies to N-CAM (Buskirk et al., 1980), and alteration of orderly mapping of the retina to the optic tectum in vivo by anti-N-CAM fragments (Fraser et al., 1984). These observations suggest a primary role for CAMs in linking cells in the proper sequence during morphogenesis and in stabilizing formed structures. We shall show later that CAM expression occurs at tissue borders and that CAM linkage is involved both in the ordering of cell movement and in the response to inductive signals. That mechanical disruption of signaling between two different tissues can affect CAM expression is seen in the example of muscle–nerve regeneration mentioned previously (Daniloff et al., 1986b).

These observations that perturbation of binding leads to morphological change and that perturbation of morphology alters CAM expression suggest that a complex interaction occurs across several levels of cellular organization to affect the primary processes that lead to form. This is not only the case for CAMs but also can involve cellular binding to various SAMs. We shall pick an interactive pair of SAMs (cytotactin and CTB proteoglycan) in order to illustrate this point later and also show that such molecules act in a coordinate fashion with CAMs during critical stages of morphogenesis. Regulation of the order of appearance of these different families of morphoregulatory molecules appears to be critical to cell and tissue patterning, and in general, form is not achieved through CAM action alone. It is therefore important to examine particular examples of coordinate regulation of members of these different families.

SAMs and CAMs: Coregulation in Morphogenesis. As already mentioned, CAMs and SAMs cannot be controlled in exactly the same fashion: CAMs are generally homophilic and are controlled by the cells they ligate, whereas adhesion via SAMs involves interactions between cell surface receptors [or integrins (Hynes, 1987; Ruoslahti & Pierschbacher, 1987)] and one or more extracellular molecules. Indeed, extensive analysis of SAM molecules such as fibronectin (Yamada, 1983) indicates a *multiplicity* of cellular binding sites as well as binding sites for other extracellular matrix molecules. So far, as many as 30 or more such molecules or their variants have been identified [see Edelman and Thiery (1985)]. What are their functions and how are they related to CAMs?

To consider this question for such a wide variety of molecules would go beyond the scope of this review. I shall consider

here only one particularly striking example, chosen because of its undoubted correlation with local cellular patterning. I refer to the recently characterized SAM (Grumet et al., 1985) called cytotactin [also known as myotendinous antigen or tenascin or hexabrachion (Chiquet & Fambrough, 1984; Erickson & Iglesias, 1984)] as well as its ligand, a chondroitin sulfate proteoglycan called CTB (cytotactin binding) proteoglycan (Hoffman & Edelman, 1987). Cytotactin has been shown to affect cell movement in a fashion that is strikingly different from that of other extracellular matrix proteins such as fibronectin and laminin (Tan et al., 1987). Cytotactin is also distinctive because it appears in a series of cephalocaudal waves of expression during development (Crossin et al., 1986). These waves are reflections of the temporal growth gradients related to macromolecular synthesis that are an important part of morphogenesis.

Cytotactin is an extracellular matrix protein consisting of 220- and 200-kDa polypeptides. It is made by glia but not neurons, by somitic mesenchyme and somites, by smooth muscle cells, by immature chondrocytes and perichondrial cells, by myotendinous regions (Chiquet & Fambrough, 1984), and by various other cells (Crossin et al., 1986; Hoffman & Edelman, 1987; Hoffman et al., 1988). It binds CTB proteoglycan, which is synthesized by central nervous system neurons and, in various forms, also by nonneural cells elsewhere in the body (Hoffman et al., 1988). Cytotactin also binds fibronectin and it has been shown to bind to fibroblasts. The most striking functional effect of cytotactin is that it causes cells in culture to round up, and it also tends to inhibit cellular migration. When mixed with fibronectin, which generally supports such migration, these effects are mitigated (Tan et al., 1987).

With this background, we may now examine briefly three examples of coregulation of CAM and SAM action in the formation of particular morphological structures. I shall choose the somites (and their associated neural crest cells as they form ganglia), the formation of layers in the central nervous system (specifically the cerebellum), and the patterning of the feather, a skin appendage that is particularly revealing because of its hierarchical and periodic structure.

Somites provide excellent examples of segmentation processes during morphogenesis. They arise in development in a craniocaudal sequence from the so-called segmental plate of mesoderm. After their initial appearance, somites undergo further patterning changes, e.g., to provide tissue for the vertebrae (via the so-called sclerotome) and also to allow periodic accumulations of migrating cells from the neural crest to form ganglia. An examination of somitic mesoderm for N-CAM and N-cadherin (or A-CAM; Volk & Geiger, 1986) during the early stages of the process of segmentation has revealed a remarkable dynamic pattern of expression (Duband et al., 1987) that is correlated with somite rearrangement and segmentation. Antibody fragments to each CAM, but particularly to N-cadherin, cause dispersal of somitic cells in culture, suggesting that somite cells are linked by these CAMs.

After such segmentation, which appears to be correlated with CAM activity, the SAM cytotactin appears adjacent to the basement membrane of the posterior portion of each somite in a cephalocaudal sequence (Crossin et al., 1986). Somewhat later, the anterior portion of each somite becomes mesenchymal, forming the sclerotome. Neural crest cells that have down-regulated N-CAM (rule I) and migrated on fibronectin paths (Thiery et al., 1985a,b) enter this region of each somite; they will later reexpress N-CAM and form dorsal root ganglia. At this time of entry or just before, both fibronectin and CTB

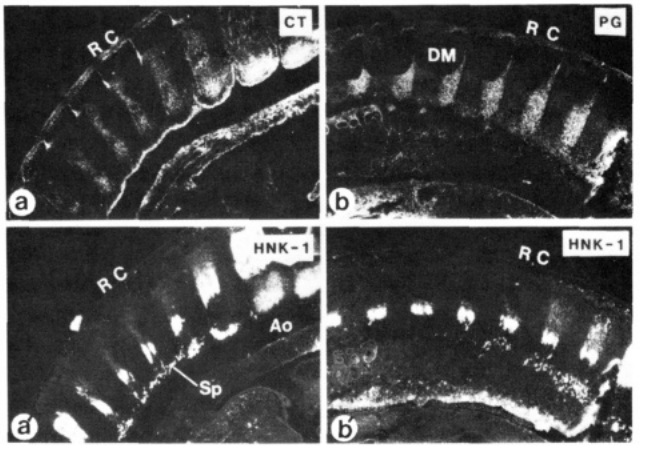

FIGURE 5: Pattern formation correlated with SAM networks. Double-labeled immunofluorescence with HNK-1 (a′ and b′) and anti-cytotactin (a) or anti-proteoglycan (b) antibodies. HNK-1 antibodies stain migrating neural crest cells. Rostral is to the left. (a and a′) Parasagittal section through a 34-somite stage embryo. Cytotactin is expressed in the rostral halves of the sclerotomes (a) and in uniform distribution along the dorsal, ventral, and intersomitic borders of the mature somites. Cytotactin is also seen surrounding the aorta. Within the sclerotome, crest cells have condensed into a compact mass in the rostral half from which the dorsal root ganglion subsequently arises (a′). In contrast, more ventrally located crest cells are evenly distributed along the wall of the aorta where they will later form the sympathetic plexus. (b and b′) Parasagittal section of a 42-somite stage embryo. CTB proteoglycan is found in a wide variety of sites except within the dermamyotome. Within the sclerotome, proteoglycan expression is polarized in the caudal half of each segment (b). Neural crest cells are localized in the rostral halves of the sclerotome (b′) but are evenly distributed near the aorta. Fibronectin, which interacts with cytotactin, is found in both rostral and caudal portions of somites (not shown). R, rostral; C, caudal; Ao, aorta; Sp, presumptive sympathetic plexus; DM, dermamyotome; CT, cytotactin staining; PG, proteoglycan staining. (Bar = 200 μm.)

proteoglycan are present throughout the somite. Cytotactin then appears (Tan et al., 1987) in just the anterior portion comprising the sclerotome (Figure 5), and after this remarkable segmented appearance, CTB proteoglycan decreases in the anterior half and is seen only in the posterior half; fibronectin remains distributed throughout.

The resultant pattern of alternating stripes of the interactive couple, cytotactin and CTB proteoglycan, is exactly correlated with the position of the neural crest cells in the cytotactin-rich anterior half and with their absence in the CTB proteoglycan rich posterior half. The concurrent finding that cytotactin alters neural crest cell shape and movement (Tan et al., 1987) is consistent with the hypothesis that, in the anterior region of each somite, cytotactin causes cell surface modulation of neural crest cells and that this cytotactin together with its ligand, fibronectin, differentially changes the pattern of motion of these cells. Clearly, this example suggests a complex regulatory sequence of patterned expression of CAMs and SAMs, showing definite correlation with cell function and distribution and the primary processes of development. The presence of a network of interactive SAMs (e.g., cytotactin, fibronectin, CTB proteoglycan) in different distributions is likely to be significant in the alteration of morphogenetic patterns.

A second example, in which the developmental sequence of CAM expression and SAM expression differentially affects processes of movement leading to cell layering, is seen in the cerebellum. In this neural tissue, layers of neurons and their neurites are formed as a result of migration of neurons called external granule cells from the exterior (pial) surface to the interior. This migration takes place on radial glial cells (Rakic, 1971) through their interaction with postmitotic neurons in

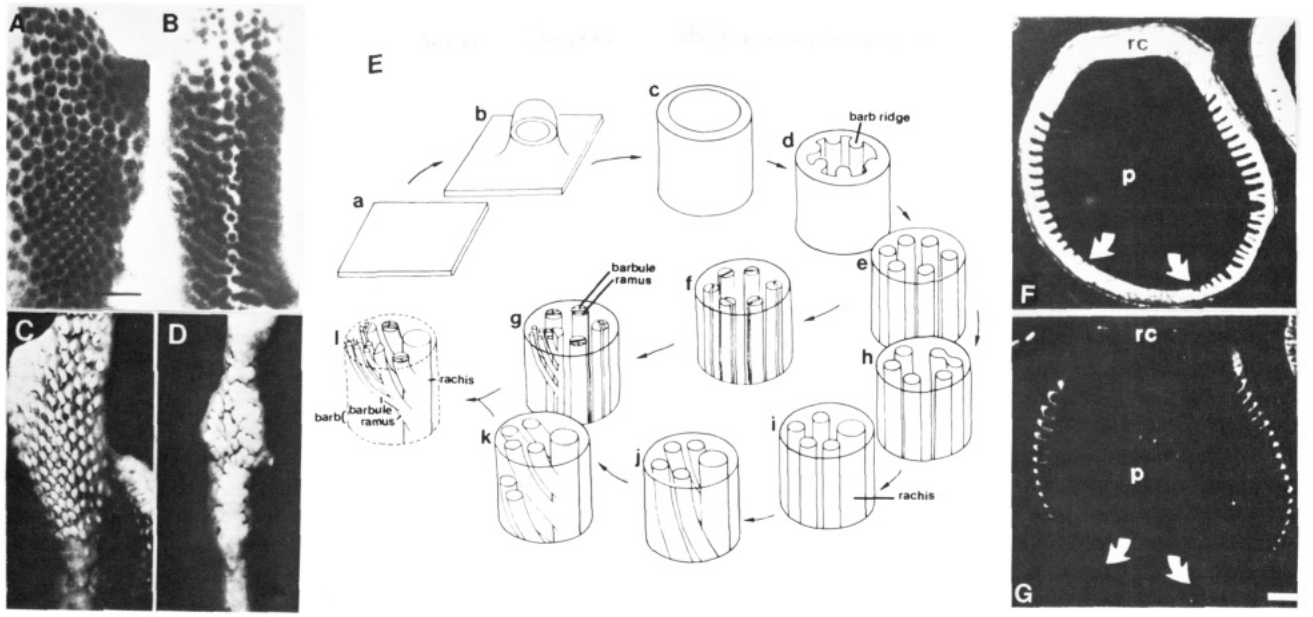

FIGURE 6: CAMs in feather induction (A–D) and histogenesis (F and G). The steps in the development of the highly ramified structure of the feather from a simple epidermal sheet are shown as a reference in the central panel (E) and will be described first. Mesenchymal cells migrate under the epidermis to induce a placode. The epidermal sheet (a) then evaginates to form a bud (b) and then a filament consisting of a cylinder of epidermis with a dermal core. The simple hollow cylinder shown in section in (c) develops ridges of epithelium (d) called barb ridges. These ridges are in turn subdivided (f, g, and l) to form the barbs and barbules that are the substance of the mature feather. The barb ridges also fuse in some places with adjacent ridges (h, i, j, and k) to form the branched pattern characteristic of the feather. (A–D) Perturbation of induction. Whole mounts of 7-day embryonic chicken skin maintained in culture for 3 (A and B) or 10 days (C and D). (A) and (C) were cultured in the presence of Fab′ fragments of antibodies from unimmunized rabbits; (B) and (D) were cultured in the presence of Fab′ fragments of rabbit antibodies against L-CAM. Note breakage of hexagonal symmetry of the mesodermal condensations [which contain no L-CAM; compare (A) and (B)] and the long-term alterations of morphology [compare (C) and (D)] induced by the antibody fragments to L-CAM. (F and G) CAMs in barb ridge formation. Transverse sections of later developing feather follicles from a newly hatched chick wing showing alternating sequence of expression of L-CAM (F) and N-CAM (G) during adult feather histogenesis. The same sections were double stained with fluorescent antibodies to each CAM. Formation of the barb ridges starts from the dorsal side (the side with the rachis, rc) and progresses bilaterally toward the ventral side, making a ventrodorsal maturation gradient. Positions of the last-formed ridges are marked by curved arrows. Fluorescent-labeled anti-L-CAM stains all the cells of the barb ridges. N-CAM appearance starts about eight ridges away from the last formed ridge and increases in staining intensity and distribution dorsally until it reaches the rachis (rc). Ultimately, all L-CAM-bearing cellular areas will become keratinized, and all N-CAM-bearing cellular areas will die. This will convert N-CAM/L-CAM borders into edges of the actual feather structure.

the so-called external granular layer. These neurons send out neurites into the subjacent molecular layer and finally translocate on the radial glia via a leading process to form the internal granule layer.

Granule cells express both N-CAM and Ng-CAM (Daniloff et al., 1986a). Addition of Fab′ fragments of anti-Ng-CAM to cerebellar tissue slices, in which movement of postmitotic cells marked with radioactive thymidine can be followed in culture, showed inhibition of external granule cell migration into the molecular layer (Chuong et al., 1987). In contrast, Fab′ fragments of anti-cytotactin antibodies had no such effect but instead caused a pileup in the molecular layer of those granule cells that had already entered that layer. Thus, a CAM of *neuronal* origin played a functional role in cell migration at an early stage (possibly by regulating fasciculation), while a SAM of *glial* origin played a different role at a later stage in the same morphogenetic process. Inasmuch as each of these molecules appears in a sequence prior to these events, there must be a precise coregulation of signals for their expression as well as coordination of their different binding functions in affecting migration. Despite the fact that anti-N-CAM Fab′ fragments had only marginal effects on granule cell migration, it is striking that the ld chain of N-CAM is specifically expressed on neurites of the molecule layer together with Ng-CAM (Chuong et al., 1987). Moreover, CTB proteoglycan, which binds cytotactin, is made by such neurons (Hoffman & Edelman, 1987). In this case, therefore, at least four molecules (and probably more), each of different provenance and type, are regulated to yield coordinate formation of cellular layers in neural histogenesis.

A final example reveals that while CAMs and SAMs must be regulated in a coordinate fashion, they have distinctly different functions in the detailed local process of cell patterning during development. This example is the feather, a hierarchically arranged and internally periodic skin appendage that itself repeats periodically in definite patterns in skin fields. Because of these properties, it is possible to examine certain roles of CAMs in the complex signaling processes of feather induction and morphogenesis, to show the differential role of CAMs and SAMs in forming borders of different feather structures, and to illustrate the cyclic expression of CAMs according to the previously mentioned rules as such borders are formed (Chuong & Edelman, 1985a,b; Crossin et al., 1985; Gallin et al., 1986). As a matter of simple convention for biochemists not trained in morphology, a few definitions may be in order. "Rostral" and "caudal" refer to head and tail ends, respectively. An epithelium consists of a sheet of cells linked by various molecules and junctions and possessing a polar structure; i.e., the basal part of the sheet rests on a basement membrane and the apical portions of the cells making up the sheet usually face the outside world or a cavity. Mesenchyme refers to cells that do not have this arrangement; mesenchymal cells are only loosely associated and can migrate.

Feathers are induced in the following fashion (Figure 6). Cells of somitic origin expressing N-CAM become mesenchymal, lose surface N-CAM, migrate to positions just below the L-CAM-positive epidermis, form mesenchymal condensations, and reexpress N-CAM (rule I). Such condensations

induce the formation of placodes, more or less circular areas of elongated cells in the epidermis. Each placode will be the site of a feather. The condensations and placodes are more or less closely packed in a hexagonal pattern that, for the dorsal feather field of the chick, begins at the midline and proceeds laterally during development. After placodes form, a feather filament is produced, and SAMs (fibronectin and laminin) form a basement membrane separating the mesodermal pulp from ectodermal structures. At this point, all further patterning to form the barbs and barbules is ectodermal.

Basilar ectodermal epithelium in the feather filament that originally expresses N-CAM and L-CAM gives rise to barb ridges expressing only L-CAM (rule II); it is these structures that will form the barbs and barbules, as well as the shaft of the feather. At a certain point in the growth of these structures, in the valley between two barb ridges, cells become N-CAM positive and L-CAM negative (Figure 6F). This process continues up each ridge to form alternating L-CAM-positive ridges, alternating with N-CAM-positive marginal plates; a similar event occurs within each barb to form barbules. After this, the L-CAM-positive ridge cells express keratin, and N-CAM-positive plate cells die, forming barbs and barbules as N–L borders are converted to edges (Chuong & Edelman, 1985a,b).

This dramatic set of patterning events illustrates the coordination of CAM expression with the primary processes of cell movement, cell division, and cell death, and it shows the cyclic expression of the primary CAMs, first in repeating feather structures and then at borders within one periodic structure. At the same time, it illustrates the different roles played by CAMs and SAMs at borders and their correlation with histogenetic events.

Perhaps the most striking result related to feather morphogenesis is one that has revealed dependent relationships among three sets of events at different levels of organization during induction: specific cell linkage by a CAM, cellular response to inductive signaling, and pattern formation (Gallin et al., 1986). Explanted 6-day-old dorsal chick skin in organ culture will show induction of a hexagonal array of feather germs. If monovalent Fab′ fragments of anti-L-CAM are added at the time of the explant, after 3 days in culture, the N-CAM-linked mesodermal condensations no longer show hexagonal patterns (Figure 6A,B) but merge into stripes with a different symmetry (Gallin et al., 1986). If the antibodies are removed and the perturbed cultures are allowed to grow for $7^1/_2$ more days, they do not show hexagonal arrays of structures that contain filaments as do unperturbed examples but instead show a cobblestone-like array of plaques with a more variable order (Figure 6C,D).

Although the N-CAM-linked cells of the inducing mesoderm that ultimately changed its pattern obviously could not be *directly* affected by the anti-L-CAM Fab′ fragments, perturbation of epidermal cell linkage by such antibody fragments nonetheless definitely altered the pattern of mesodermal condensations. This suggests that the antibody effects were indirect, altering the response to signals from the dermis as well as production of ectodermal signals, both in such a fashion as to alter the timing and control of mesodermal growth patterns. Thus, it appears that the linkage of cells by a specific CAM can regulate their ability to produce signals and respond to other signals. A computer model based on this idea has been constructed, and it gives rise to altered patterns that closely resemble the perturbed case (Gallin et al., 1986).

The findings discussed here underscore the fact that it is the complex play among primary processes of development,

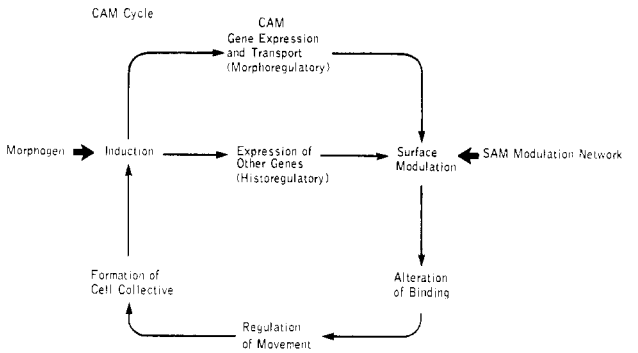

FIGURE 7: The morphoregulator hypothesis [see Edelman (1984, 1985, 1986a,b)] as exemplified in a CAM regulatory cycle. Early induction signals (heavy arrow at left) lead to CAM gene expression. Surface modulation (by prevalence changes at the cell surface, polar redistribution on the cell, or chemical changes in CAMs) alters the binding rates of cells. This regulates morphogenetic movements that in turn affect embryonic induction or milieu-dependent differentiation. The inductive changes can again affect CAM gene expression in a cycle as well as the expression of other genes for specific tissues. The heavy arrows at the left and right refer to candidate signals for initiation of induction, which are still unknown. These signals could result from global surface modulation as a result of CAM binding or binding of substrate adhesion molecules (SAMs) that act in networks as seen in basement membranes and the extracellular matrix (right) or from release of low molecular weight morphogens affecting induction (left) or both; in any case, through CAM binding, a mechanochemical link is provided between gene expression and morphogenesis. The activity of morphoregulatory genes for CAM expression is independent of the activity of historegulatory genes affecting cell products other than CAMs and SAMs.

cellular responses to signals, and the modulatory mechanochemical effects of CAMs and SAMs that gives rise to tissue pattern. Pattern is not "built in" to large ensembles of molecular binding specificities but is dynamically regulated in response to signals expressed in CAM cycles and networks of interacting SAMs.

CAM Cycles and SAM Networks: The Morphoregulator Hypothesis. The idea that cell surface modulation with transmembrane control underlies these patterning events may be incorporated into a morphoregulator hypothesis (Edelman 1984, 1986a). This states that the essential genetic component controlling pattern formation is the response of regulatory genes and structural genes determining the appearance and function of morphoregulatory molecules. These sets of genes are assumed to be under separate control from those specifying specific tissue products (Figure 7), and they respond to signals that arise across borders formed between different collectives of cells linked by CAMs of different specificities. The appearance of these CAMs and the action of networks of SAMs that form different cell surface modulating combinations at different sites by binding to each other and to cell receptors alter the growth and movement of these cells. Thus, the successive synthesis, turnover, or degradation of CAMs and SAMs leads to new contexts for inductive signaling resulting in further gene expression altering borders and subsequent signaling events. The number of such signals need not be large. The various CAMs, with their cyclic expression and modulation mechanisms as well as the combinatorial effects on modulatory functions of different interactive SAMs occurring in different amounts, could combine with the general irreversibility of previous gene expression events to yield a large number of potential patterns. Such morphoregulatory sequences are essential but could not in themselves lead to detailed tissue pattern. We must assume in addition that, at the same time, batteries of developmentally important *tissue-specific* genes are controlled by selector genes resembling

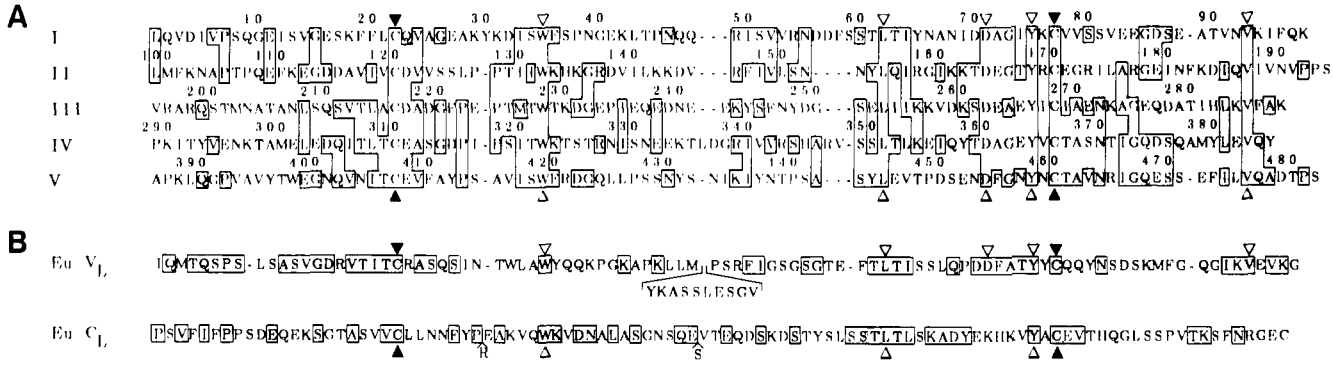

FIGURE 8: Internal homology of N-CAM domains and homology with the light chain of IgG (Eu). (A) Alignment of the five internally homologous segments (I–V) common to the ld, sd, and ssd chains showing their similarity to each other and to members of the Ig superfamily (one-letter amino acid code). Residues are numbered consecutively from the amino terminus of the mature N-CAM polypeptides. Residues identical in all five N-CAM regions and highly conserved among Ig-like proteins are marked with triangles; the cysteines proposed to be involved in intradomain disulfide bonds are indicated by closed triangles. Residues identical in two or more sequences are boxed. (B) Similar comparison to the V and C regions of IgG (Eu).

homeotic genes [see Scott and O'Farrell (1986)] to yield sequences of *tissue differentiation* within the patterns established by morphoregulatory genes.

The morphoregulator hypothesis links developmental genetics to the mechanochemistry of pattern by connecting CAM and SAM modulation in cell collectives to gene expression, correlating the ensuing signals and responses of the cell collectives that emerge with particular states of adhesion. Although I have not stressed the functions of CJMs (for example, in tight junctions, adherens junctions, and gap junctions) in this paper, it is clear that the general picture sketched here also applies to them. It seems highly likely, however, that their expression and regulation, governing cell communication and epithelial stabilization, will depend upon the prior expression and action of CAMs. This is also true for CAMs and SAMs in early development, and it may be proposed that the precedence order necessary to form fully functioning epithelia is (1) CAMs, (2) SAMs, and (3) CJMs. This precedence hypothesis is subsidiary to the morphoregulator hypothesis and is more immediately open to test.

Evolutionary Implications. The idea of a dynamic connection between cell adhesion, morphogenesis, and gene signaling has several implications both for the evolution of form and for the emergence of various functional specialized tissue systems. It is known that SAMs are found in both vertebrates and invertebrates (Fessler et al., 1984). Moreover, although definitive invertebrate CAMs have not yet been identified, it seems likely that they exist and that their function will be correlated with the control events currently being dissected, for example, through the analysis of homeotic and segmentation genes in *Drosophila* (Scott & O'Farrell, 1986). In the vertebrates, N-CAM binding functions have been shown to be highly conserved (Hoffman et al., 1984). Furthermore, although a comparison of chick and frog primary CAM expression (Levi et al., 1987) has shown preservation of the CAM expression rules, it strongly suggests that there are large differences between species in the timing and extent of gene expression for a given CAM at comparable stages of development and morphogenesis. Thus, it may be that morphological changes seen in evolutionarily related forms as well as in metamorphosis occur as a result of molecular heterochrony—differential alterations in the timing of expression of homologous modulatory CAMs and SAMs during evolution of developmental morphogenetic sequences. This has the appeal of genetic parsimony. Despite the ultimate involvement of large batteries of genes in development, according to the morphoregulator hypothesis, not too many genes would have to be altered to yield relatively large morphogenetic changes even at early developmental stages, inasmuch as many of the heterochronic changes would involve morphoregulatory genes, i.e., those controlling the appearance of morphoregulatory molecules.

Another intriguing aspect of molecular histology has been revealed by the study of morphoregulatory molecules, providing an example of the emergence in evolution of one tissue function from another. It appears that structural genes specifying N-CAM are closely related (Hemperly et al., 1986a,b; Cunningham et al., 1987) to the precursor for the highly versatile group of proteins comprising the immunoglobulin superfamily. As shown for one example in Figure 8, N-CAM homology regions are closely related to Ig domains. They have the length of C regions but are more closely homologous to V regions, and each disulfide-linked loop, unlike those of the Ig's, is specified by two exons. It thus appears likely that the adaptive aspects of cellular and humoral immunity that relate to self–non-self-recognition may have emerged from early origins in the self–self recognition of cell adhesion (Edelman, 1987). Moreover, several other types of molecules including certain growth factor receptors, proteoglycan link proteins, and nervous system specific molecules such as myelin-associated glycoproteins (MAG; Arquint et al., 1987; Salzer et al., 1987) and the Po myelin-associated glycoproteins (Lemke & Axel, 1985) also share this evolutionary origin. It would not be surprising if other secondary CAMs in the nervous system such as Ng-CAM were found to be homologous to Igs.

The evolutionary relationship of cell adhesion to adaptive immune responses is perhaps not surprising in the sense that both morphoregulation and immune regulation require cell–cell interactions at the cell surface. The opportunistic character of evolution is no better seen than in this example, in which genes, originally established for metazoan transcendence over the life of the solitary cell, are used subsequently as the basis in evolution for genes mediating some of the most sophisticated molecular recognition phenomena yet analyzed. Undoubtedly, the further study of morphoregulatory molecules will provide other examples and additional insights into the evolution of highly specialized tissue functions. But above all, the study of families of these proteins will transform our view of anatomy and development and of their relation to evolution, providing the basis for a science of molecular histology. Any static picture of biochemistry, suggesting that it simply accounts for energetics in solution, for specificity in molecular interactions, and for strengths of supramolecular structures, is incomplete. Instead, the dynamic picture is emerging that anatomy, upon

which animal function rests and natural selection acts, is in fact regulatory biochemistry.

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