NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

UNIVERSITY OF ALBERTA

NEURAL CELL ADHESION MOLECULE (N-CAM) IN DEVELOPING FEATHERS: ANALYSIS OF STRUCTURAL VARIANTS AND PERTURBATION OF FUNCTION

by

Randall Glenn Marsh

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

Edmonton, Alberta
Spring 1992

ISBN 0-315-73157-5

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: Randall Glenn Marsh

TITLE OF THESIS: Neural Cell Adhesion Molecule (N-CAM) in Developing Feathers: Analysis of Structural Variants and Perturbation of Function

DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: 1992

Randall G Marsh

Randall Glenn Marsh
9751 - 146 Street
Edmonton, Alberta
T5N 2Z3

Date: April 23, 1992

We must not conceal from ourselves the fact that the causal investigation of organisms is one of the most difficult, if not the most difficult problem which the human intellect has attempted to solve, and that this investigation, like every causal science, can never reach completeness, since every new cause ascertained only gives rise to fresh questions concerning the cause of this cause.

WILHELM ROUX (1894)

Seek simplicity and distrust it.

ALFRED NORTH WHITEHEAD (1919)

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

Neural Cell Adhesion Molecule (N-CAM) in Developing Feathers: Analysis of Structural Variants and Perturbation of Function

submitted by Randall Glenn Marsh
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE.

Warren Gallin
(Dr. W. J. Gallin, Supervisor)

Sara E. Zalik
(Dr. S. E. Zalik, Examiner)

Marijeh Pasdar
(Dr. M. Pasdar, External Examiner)

Norm Stacey
(Dr. N. E. Stacey, Chairman & Examiner)

Date: April 20, 1992

ABSTRACT

The neural cell adhesion molecule (N-CAM) is a family of proteins that mediate cell-cell contact. The individual N-CAM proteins may have different functions in regulating cell-cell interaction during development, but these differences are not understood. A first step in understanding the functions of the different forms of N-CAM is to correlate the expression of these forms with different morphogenetic events. This thesis describes (a) the structural forms of N-CAM present in developing chicken feathers and, (b) the effects of perturbing N-CAM-mediated adhesion in this system.

N-CAM in skin appears as a 145 kD protein, encoded by a 6.4 kb mRNA, compared to the 140 kD N-CAM protein and 6.1 kb mRNA in brain. PCR analysis shows the 6.4 kb skin mRNA actually consists of two transcripts, with and without a 93-bp insert between exons 12 and 13. Because only a single mRNA band is detected in skin, the 93-bp insert can not be the basis for the 300-bp larger size of the two skin mRNAs. Their larger size may be due to use of an alternative transcriptional start site. The presence of two mRNAs means that two polypeptides are probably expressed in skin. However, because only a single protein is detected, the 93-bp insert can also not account for the 5 kD larger size of the skin proteins. Their larger size is due to N-linked carbohydrate. Localization of N-CAM and polysialic acid (PSA) in skin reveals that N-CAM is present on epidermal cells as early as E6 and that PSA is expressed at specific times on only a subset of the cells that express N-CAM.

Perturbation of N-CAM-mediated adhesion using an antibody that inhibits N-CAM homophilic binding, and removal of PSA using endoneuraminidase-N, did not cause any apparent changes in formation of feather rudiments or filaments.

In summary, variations in N-CAM mRNA and protein, which may be important in modulating N-CAM function in developing feathers, were discovered. Initial analyses of the effects of varying some of these factors did not provide any insight into the function of these variations.

PREFACE

Embryogenesis in vertebrates proceeds through a series of cellular interactions in which different cells become sorted out and assembled into multicellular groupings that give rise to tissues and organs. The capacity of cells to recognize one another, adhere, and assemble into groups during development depends on interactions between cell surface constituents on apposed cells. Cell adhesion molecules (CAMs) are cell surface proteins that mediate direct contact between the plasma membranes of apposed cells.

Cell adhesion mediated by CAMs is involved in the formation of intercellular junctions, which allow for communication between cells and for the development of physiologically distinct tissue compartments. The formation of intercellular junctions is thought to join cells into groups that exhibit characteristics different from those of single cells. These collectives of cells respond to and generate inductive signals that cause changes in gene expression and subsequently lead to differentiation and the establishment of form. Thus, the processes that are involved in cell adhesion are of prime importance in development, and characterization of these processes should provide insight into how development occurs.

The neural cell adhesion molecule (N-CAM) is a family of proteins that mediate direct cell-cell contact between a variety of cells in the developing embryo. The different N-CAM proteins are generated from a single gene by differential mRNA splicing. The individual N-CAM proteins are expressed at specific times and locations in the

embryo, and are often present on cell groups during inductive interactions. This suggests that the individual N-CAM proteins may have different functions and may play specific roles in inductive interactions during development.

The function of the N-CAM proteins is thought to be modulated by attachment of a specialized carbohydrate moiety called polysialic acid (PSA). PSA is large and negatively charged, and decreases N-CAM-mediated adhesion by preventing contact between plasma membranes of apposed cells. The amount of PSA attached to N-CAM is regulated during development, suggesting that PSA may play an important role in controlling N-CAM-mediated adhesion during morphogenesis. At present, the developmental function of PSA, and of the different N-CAM proteins, is poorly understood.

Feather formation in chicken skin offers a model in which to examine the function of N-CAM-mediated adhesion in the formation and patterning of embryonic cell populations, i.e. the collectives that are thought to be the basic units of development. Feather morphogenesis is characterized by the formation of discrete epidermal and dermal cell groups that interact with one another and differentiate into the mature feather. During feather formation, N-CAM is known to be expressed on cells that are involved in epidermal-dermal induction, on cells that are undergoing division, and on cells that may undergo programmed death. This suggests that differential control of N-CAM-mediated adhesion by selective expression of the N-CAM proteins and of PSA may be involved in regulating these cellular events and in directing feather formation.

This thesis describes the structural forms of N-CAM present, and the effects of perturbing N-CAM-mediated adhesion, during feather morphogenesis. As a background to describing this work, the introduction contains a historical summary of the field of cell adhesion and how N-CAM was isolated, a description of the features of N-CAM and how its adhesive properties are regulated, an overview of the functions that N-CAM-mediated adhesion may have in development, and a description of feather formation and N-CAM expression during this process. The following two sections of the thesis describe the forms of N-CAM present in developing feathers and a preliminary investigation into the effects of perturbing N-CAM-mediated adhesion in this system. The final section is a discussion of these results in terms of possible functions of N-CAM-mediated adhesion in developing feathers and future directions for research.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my research supervisor, Dr. Warren Gallin, for working with me on all aspects of this project. He served as a constant impetus to get work done and, most importantly, taught me how to think and write logically.

I would also like to thank the other members of my supervisory committee, Drs. Ann Acheson and Sara Zalik, for their valuable criticisms, and for serving as a source of information and intellectual stimulation. As well, I am grateful to the students and staff of the Department of Zoology for their friendship and support during the last several years.

Finally, I would like to give special thanks to my wife, Sandy, for her constant support and encouragement, and to my parents, who were never quite sure what I was working on, but were always confident it would turn out fine.

The anti-PSA 5A5, anti-N-CAM 5e, and anti-tropomyosin CH1 mAbs were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD.

Materials for preparation of endoneuraminidase-N were kindly supplied by Dr. Eric Vimr, Department of Veterinary Pathology, University of Illinois at Urbana-Champaign.

This work was supported by grants from the Natural Sciences and Engineering Research Council, the Medical Research Council, and the Alberta Heritage Foundation for Medical Research. R.G.M. was supported by NSERC and AHFMR Postgraduate Studentships.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER III

ABBREVIATIONS

bp	base pair
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	bovine serum albumin
CAM	cell adhesion molecule
ChAM graft	chorioallantoic membrane graft
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
DAB	3,3' diaminobenzidine tetrahydrochloride
DMSO	dimethyl sulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
endo-F	endoglycosidase-F
endo-N	endoneuraminidase-N
FITC	fluorescein isothiocyanate
FLS	filament-like structure
HNK-1	human natural killer cell 1
HRP	horseradish peroxidase
kb	kilobase
kD	kilodalton
L-CAM	liver cell adhesion molecule
mAb	monoclonal antibody
MSD1	muscle specific domain 1
M_r	relative molecular weight
NBT	nitro blue tetrazolium
N-CAM	neural cell adhesion molecule
PBS	phosphate buffered saline

PMSF	phenylmethanesulfonyl fluoride
PNA	peanut lectin
PSA	polysialic acid
RACE	rapid amplification of cDNA ends
RITC	rhodamine isothiocyanate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris buffered saline
TTBS	TBS with 0.1% Tween 20
VASE (π)	variable domain alternatively spliced exon
WGA	wheat germ agglutinin

CHAPTER I

History and Background

Historical summary of the field of cell adhesion

The central problem of developmental biology is to understand the mechanisms by which form is generated and maintained. Early descriptive studies of development in different animals show that embryogenesis proceeds through a series of interactions in which different cells sort-out and assemble into multicellular groupings that give rise to tissues and organs. Each organ has a distinct form, a precisely ordered arrangement of cells, and specific relationships to other organs. Clearly, certain mechanisms must be responsible for holding cells together to allow for segregation and organization of cell groups in the developing embryo, and must also be involved in stabilizing multicellular structures once they have formed.

The first experimental approach to understanding how cells recognize and adhere to one another is attributed to Wilson (1910), who dissociated sponges by forcing them through silk mesh. The dissociated cells re-aggregate into clusters that eventually form into new, intact sponges. Wilson observed that reformation of new individuals depends upon movement of cells within the aggregates so that their appropriate position within the mass is regained. He had thus discovered the ability of cells to self-assemble into structures having a higher level of organization. Later experiments conducted by Galtsoff (1925) show that cells from different sponge species

preferentially aggregate with their own type in a mixture of cells from two species, demonstrating that specific recognition occurs at the level of the individual cell.

Further evidence that species-specific re-aggregation is due to adhesion of cells to their own kind came from the use of a rotary shaker method developed by Moscona (1961). This method allows cell re-aggregates to form as a result of adhesive stability only, and rules out the potential for cell migration that may occur in stationary (non-rotated) systems. When mixtures of different sponge species are rotated together using this method, aggregates consisting of only one cell type are still formed. This confirms the idea that each cell preferentially adheres to its own kind and that recognition occurs at the level of the single cell.

The ability of cells to selectively recognize and adhere to one another is not only a property of invertebrate cells; when cells of developing vertebrate embryos are mixed, they too segregate according to cell type and re-establish their former associations. Townes and Holtfreter (1955) found that when dissociated cells from a whole amphibian embryo or from various tissues are incubated in tissue culture dishes, the cells recombine into aggregates containing several cell types. After several days, cells in these aggregates sort-out into defined groups and form individual cell populations composed of only one cell type. By using embryos from species having cells of different sizes and colors, the behavior of the recombined cells can be followed. Cells within the aggregates are observed to sort-out into defined regions in accord with their original position in the embryo, i.e. epidermal cells outside, mesoderm in the

middle, and endodermal cells inside. In addition, the adhesive affinities of each cell population, and hence their position within the aggregate, is found to change at different developmental stages. This implies that specific interactions between cell populations occur during embryogenesis.

In order to explain the sorting-out of cells seen during development, Steinberg (1970) proposed a model for adhesive interaction that is based on free energy of cell-cell binding. This theory, called the differential adhesion hypothesis, suggests that mixed cells interact to form an aggregate by rearranging themselves into a thermodynamically stable pattern with low free energy. The only requirement for the sorting to occur is for cells to differ in their relative strengths of adhesion. By varying either the amount or type of cellular "glue," or both, the stability of contact between cells can be altered and cells can sort-out due to thermodynamic differences in binding. In theory, the use of relatively few adhesion molecules should be sufficient to allow for sorting and patterning of cell populations during embryogenesis, since each population can preferentially adhere to another by changing the quantity or type of "glue" at specific times and places.

While all of these observations and ideas argue that selective adhesion between cells plays a role in the sorting-out of cell populations during development, none identify the molecules underlying this process. It is not until the late 1970s that the molecular basis for cell-cell adhesion would start to be identified.

The strategy used in searching for molecules involved in cell adhesion is based on an immunological approach in which antisera are produced that block adhesion between cells. The basic idea is that when an antiserum containing an antibody to an adhesion molecule is incubated with cells, it binds to the adhesion sites on the cell surface and blocks adhesion between the cells. Brackenbury et al. (1977) used this idea to raise antisera against cells from chicken neural retina and isolate antisera that inhibit cell-cell aggregation. To purify the specific anti-cell adhesion molecule (CAM) antibodies in the antisera, a neutralization assay is used in which fractions of cell surface proteins are tested for their ability to compete with cells for binding to the anti-CAM antibody. Fractions that prevent binding of cells to the antibody must contain the original CAM that the antibody binds to. These fractions are used to re-immunize rabbits and generate new antisera. By iteration of this procedure, pure antigenic fractions are obtained and used to generate specific anti-CAM antibodies. This type of approach led to the isolation of the neural cell adhesion molecule (N-CAM) (Brackenbury et al., 1977; Thiery et al., 1977), liver cell adhesion molecule (L-CAM) (Bertolotti et al., 1980; Gallin et al., 1983), and neural-glial cell adhesion molecule (Ng-CAM) (Grumet and Edelman, 1984; Grumet et al., 1984a). The remainder of this introduction describes the features and developmental functions of the neural cell adhesion molecule, N-CAM.

Structural characteristics of N-CAM and modulation of its adhesive properties

N-CAM is a family of closely related proteins that mediate calcium-independent, homophilic adhesion between a variety of cell types in the developing embryo (Hoffman et al., 1982; Thiery et al., 1982; Crossin et al., 1985; Hall et al., 1990). In embryonic chicken brain there are three major N-CAM polypeptides with molecular weights of 180, 140, and 120 kD (Murray et al., 1986) (see Fig. I-1). All three proteins are identical from their amino terminus, which is outside the cell, to the region where they are associated with the cell membrane (Murray et al., 1986; Cunningham et al., 1987). This extracellular region of the proteins contains five immunoglobulin-like domains in which the binding site for N-CAM is located; interaction between these domains on two apposed N-CAM molecules forms the basis of N-CAM homophilic binding (Cunningham et al., 1987; Hall et al., 1990). The two larger N-CAM polypeptides are integral membrane proteins and have cytoplasmic domains. The larger of these (180 kD) contains an additional 261 amino acids in its cytoplasmic domain (Cunningham et al., 1987). The smallest polypeptide (120 kD) lacks a membrane spanning region entirely, and is attached to the cell membrane via a phosphatidylinositol lipid anchor (Sadoul et al., 1986; Hemperly et al., 1986b; He et al., 1987).

The different N-CAM polypeptides are generated by alternative splicing of a single gene (Murray et al., 1986) (see Fig. I-2). The N-CAM gene in chicken is approximately 110 kilobases (kb) in length and contains 19 exons (Murray et al., 1986; Hemperly et al., 1986a; Cunningham et al., 1987; Colewell et al., 1991). The first fourteen

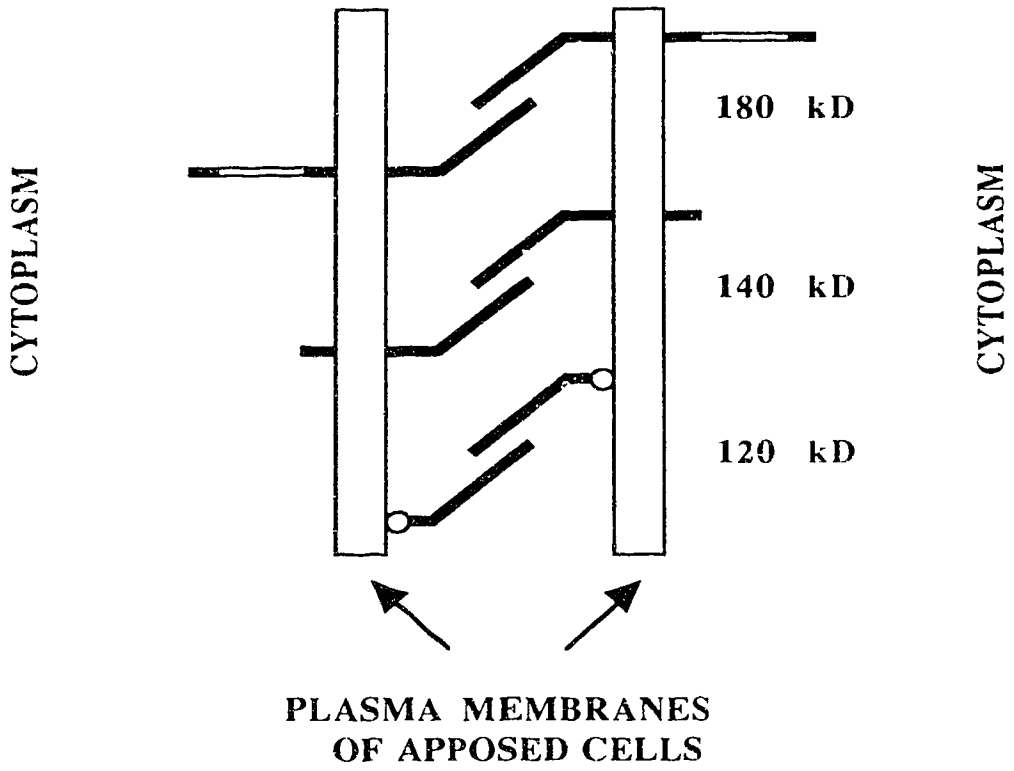


Fig. I-1. Diagramatic representation of the three major N-CAM polypeptides. All three are identical from their amino terminus (outside cell) to the region where they are associated with the plasma membrane. The 180 kD and 140 kD polypeptides are both transmembrane, but the 180 kD polypeptide contains an extra cytoplasmic domain (═══). The 120 kD polypeptide is attached to the plasma membrane by a phosphatidylinositol anchor (O). Interaction between the extracellular domains of the polypeptides on apposed cells forms the basis of N-CAM homophilic binding.

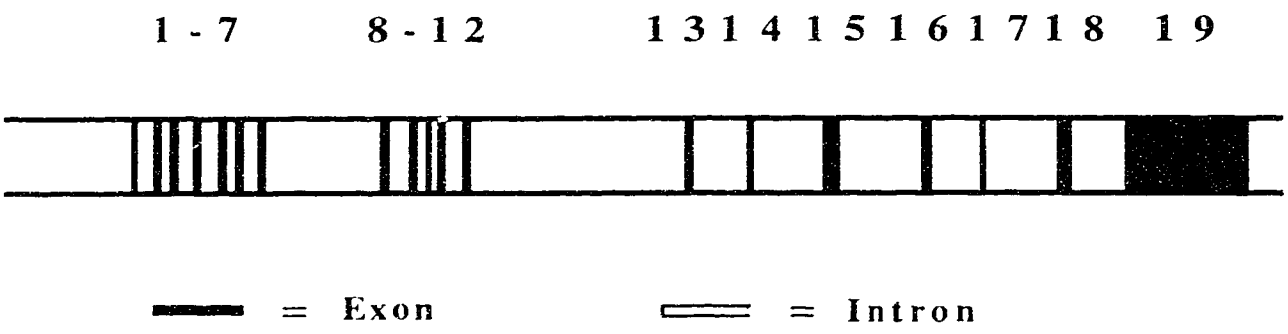
exons of the gene are common to all of the N-CAM proteins; the differences in the individual protein chains arise by alternative splicing of the remaining five exons. Exon 18 is specific to the 180 kD protein and encodes the extra cytoplasmic domain present in this form (Murray et al., 1986). Exon 15 is present only in the 120 kD polypeptide and generates the phosphatidylinositol linkage (Murray et al., 1986; Hemperly et al., 1986b). The size of the mRNAs generated for the brain N-CAM proteins are 6.9, 6.1, and 4.2 kb for the 180, 140, and 120 kD forms, respectively (Murray et al., 1986). These mRNA and protein sizes are specific for brain. Other tissues in the chicken embryo contain different-sized N-CAM mRNAs and polypeptides due to alternative splicing of additional exons and to glycosylation of the proteins, as explained below.

The adhesion mediated by N-CAM is regulated in several ways during development, namely (i) changes in the amount of N-CAM expressed at the cell surface, (ii) changes in the N-CAM proteins being expressed due to differences in mRNA splicing, and (iii) post-translational modification of the N-CAM polypeptides.

(i) Changes in the amount of N-CAM at the cell surface

Variation in the amount of N-CAM present on the cell surface causes large changes in the rate of N-CAM homophilic binding (Hoffman and Edelman, 1983). Membrane vesicles prepared from brain tissue, or vesicles reconstituted from purified N-CAM and lipid, both show large changes in the rate of N-CAM binding (vesicle aggregation) proportional to the surface density of N-CAM. Specifically, aggregation rate of the vesicles increases in proportion

A. Gene Structure



B. Differential Splicing of mRNA

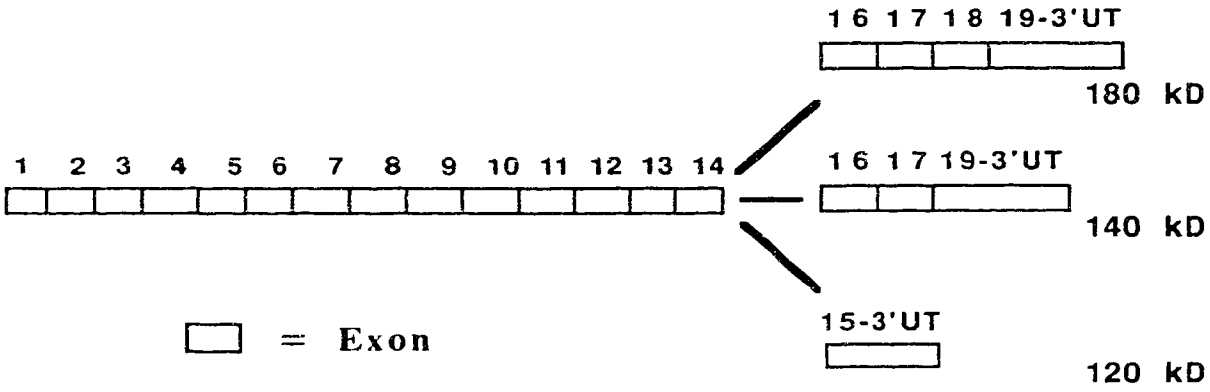


Fig. I-2. Diagramatic representation of (A) the structure of the N-CAM gene and (B) the alternative splicing that generates the mRNAs for the 180, 140, and 120 kD N-CAM polypeptides. The N-CAM gene (A) contains 19 exons, which are differentially spliced (B) to produce the three major N-CAM polypeptides. The first 14 exons are common to all of the N-CAM polypeptides. Exon 18 is present only in the 180 kD form, and exon 15 is present only in the 120 kD form. 3'UT represents the 3' untranslated region of the mRNAs.

to the 4th power of N-CAM concentration. This suggests that the developmental function of N-CAM may be modulated by variations in its prevalence. Consistent with this idea is the observation that neural crest cells decrease expression of N-CAM during migration but re-express N-CAM concomitant with their aggregation and differentiation into ganglia (Thiery et al., 1982; Edelman et al., 1983).

(ii) Changes in the N-CAM polypeptides due to alternative mRNA splicing

Another mechanism involved in regulating N-CAM-mediated adhesion is spatiotemporal variation in the N-CAM polypeptides being expressed. Immunostaining of N-CAM in the chicken embryo shows that the different N-CAM polypeptides are expressed in specific patterns during morphogenesis of individual tissues (Pollerberg et al., 1985; Daniloff et al., 1986; Moore et al., 1987). For example, the 180 kD protein is expressed only in tissues of the central and peripheral nervous system, and different regions of the brain each express varied amounts of the 180, 140, and 120 kD polypeptides during development (Chuong and Edelman, 1984; Pollerberg et al., 1985; Daniloff et al., 1986; Prieto et al., 1989). Similarly, in embryonic heart and skeletal muscle, a shift from the 140 kD to the 120 kD N-CAM polypeptide occurs concomitant with myoblast fusion (Covault et al., 1986; Wharton et al., 1989). These, and other expression patterns of the individual N-CAM proteins, suggest that the polypeptides may have different functions in development. These differences in function, however, are not well understood.

In addition to the three major N-CAM polypeptides, several additional variants of the polypeptides exist (Dickson et al., 1987; Prediger et al., 1988; Small et al., 1988; Santoni et al., 1989; Thompson et al., 1989; Small and Akeson, 1990; Reyes et al., 1991; Zorn and Krieg, 1992). These variations are due to splicing of small, extra exons into the N-CAM mRNAs, leading to the insertion of additional peptide sequences within the N-CAM proteins. The extra exons spliced into the mRNAs are present within the N-CAM gene in large introns between exons 7-8 and 12-13. Consequently, these exons are inserted into the mRNAs between the junctions of these exons (see Fig. I-3). Several different splices are known for rat, human, and frog N-CAM, but the only alternative splice known in chicken is a 93-bp insert present in skeletal muscle and heart N-CAM mRNA between exons 12-13 (Prediger et al., 1988) (see Fig. I-3).

The alternative exons are expressed in specific spatiotemporal patterns, both within given tissues and in different developing tissues. For example, in some areas of rat brain, the 30-bp VASE (or π) exon (see Fig. I-3) is present in all of the N-CAM proteins, while in other areas there is mixture of N-CAM with and without it, and in some brain regions N-CAM does not contain VASE at all (Small and Akeson, 1990). In a more extreme case, embryonic rat heart contains at least 27 different-spliced forms of N-CAM generated by alternative splicing of the VASE exon and subsets of the four exons that comprise the MSD1 insert (see Fig. I-3), in conjunction with the splicing that produces the 140 and 120 kD N-CAM polypeptides found in heart (Reyes et al., 1991). This complex splicing pattern varies during heart formation, with several of the different splicing

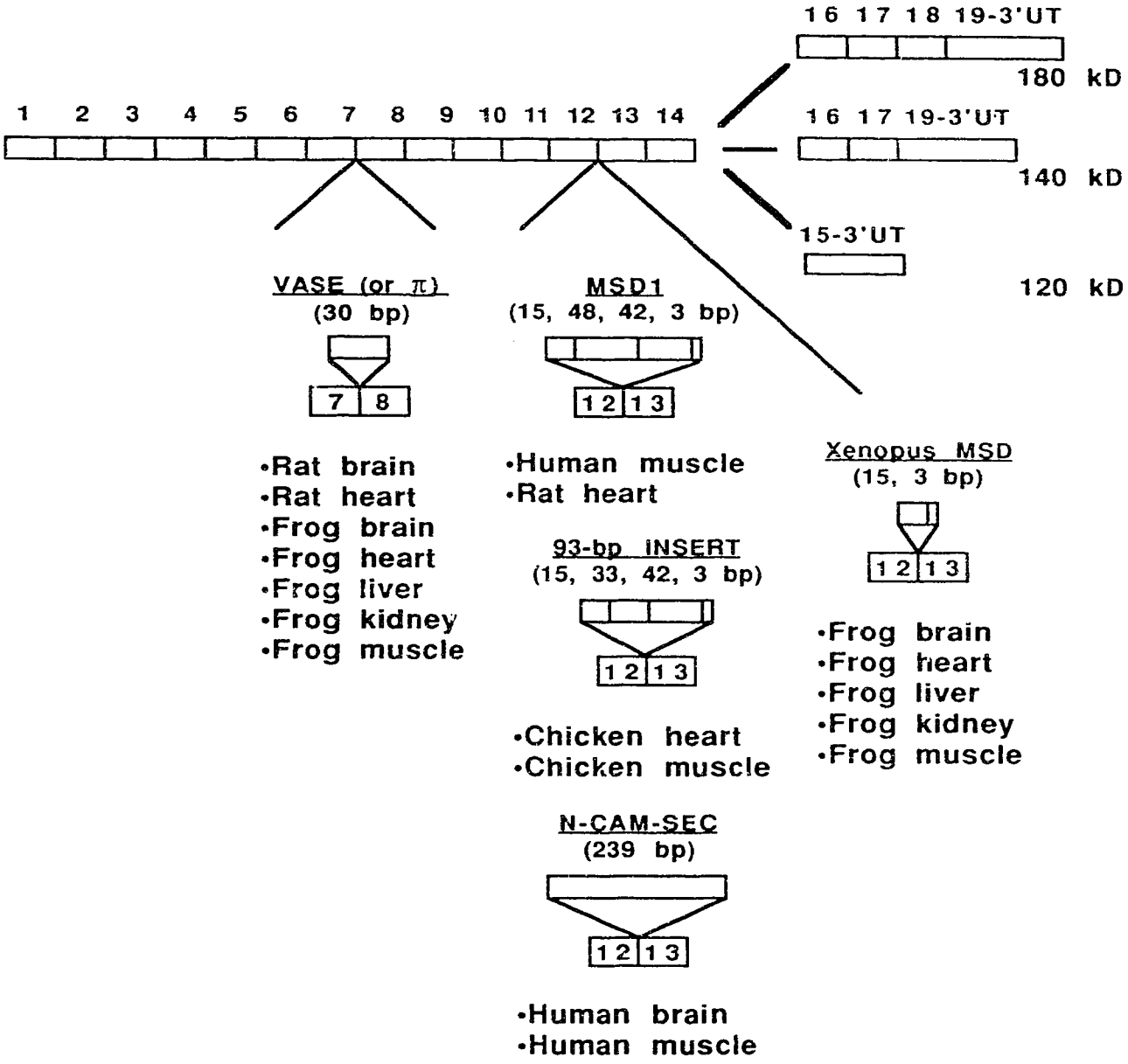


Fig. I-3. Alternative exons spliced into chicken, human, rat, and frog N-CAM mRNA at the junctions of exons 7-8 (VASE or π) and exons 12-13 (MSD1, 93-bp insert, N-CAM SEC, Xenopus MSD). Each box represents an individual exon. Boxes labelled 1-19 represent the exons that are spliced to generate the 180, 140, and 120 kD polypeptides. 3'-UT represents the 3' untranslated region of the mRNAs.

combinations being preferentially expressed at any given stage. These observations suggest that extremely fine control of N-CAM-mediated adhesion may be involved in the morphogenesis of individual tissues.

The functional significance of the alternative exons is poorly understood. Both the chick 93-bp and human MSD1 insert have a high content of proline, serine and threonine (Prediger et al., 1988). The proline residues could introduce a hinge region that gives the N-CAM polypeptides extra flexibility and enhances binding, but this is unknown. The serine and threonine residues, however, are known to serve as sites for O-linked glycosylation in the MSD1 insert (Walsh et al., 1989). As well, the N-CAM-SEC insert (see Fig. I-3) is known to introduce an in-frame stop codon that generates a truncated form of N-CAM which is secreted (Gower et al., 1988). This secreted form of N-CAM may act as a soluble modulator of cell-cell adhesion or cell-substratum interaction as a component of the extracellular matrix (Probstmeier et al., 1989).

The mechanisms regulating the complex stage- and cell-type-specific expression of N-CAM are also poorly understood. The promoter regions for the N-CAM gene have only recently been isolated (Chen et al., 1990 - rat; Hirsch et al., 1990 - mouse; Colewell et al., 1991 - chicken). In all of these animals the N-CAM promoter does not contain a typical transcriptional start site, and transcription of the N-CAM gene instead begins at several sites that may be used differently in individual tissues (Barthels et al., 1987; Chen et al., 1990; Hirsch et al., 1990; Colewell et al., 1991; Brackenbury, 1992).

At the other end of the gene, there is evidence that alternative polyadenylation sites are also used (Goridis and Wille, 1988). At present, the function of using different start sites and polyadenylation signals is not known.

(c) Post-translational modification of the N-CAM polypeptides

In addition to the variation in N-CAM-mediated adhesion generated by alternative splicing of the major polypeptides and extra exons, a variety of post-translational modifications may also regulate N-CAM's adhesive properties. Some of the modifications, such as sulfation of N-linked carbohydrates on the proteins (Sorkin et al., 1984) and phosphorylation of amino acids in the cytoplasmic domains (Gennarini et al., 1984; Lyles et al., 1984; Sorkin et al., 1984) have unknown effects on N-CAM. However, other modifications such as the addition of N-linked carbohydrates to the N-CAM proteins (Hoffman and Edelman, 1983; Cole and Schachner, 1987; Kunemund et al., 1988) do have known effects on N-CAM-mediated adhesion.

As with the expression of the individual N-CAM proteins, glycosylation of the polypeptides is also regulated in a specific spatiotemporal manner (Cunningham et al., 1983; Crossin et al., 1984; Walsh et al., 1989; Key and Akeson, 1990, 1991). There are several potential sites for N- and O-linked carbohydrate attachment on N-CAM (Lyles et al., 1984; Barthels et al., 1987; Cunningham et al., 1987; Barton et al., 1988; Nybroe et al., 1988), but not all of these sites are used (Crossin et al., 1984; Cunningham et al., 1987). Several different N-linked carbohydrates are known to attach to these sites, including HNK-1 and polysialic acid (Rothbard et al., 1982; Kruse et

al., 1984; Key and Akeson, 1990, 1991), but only one O-linked carbohydrate is known to attach to N-CAM, this carbohydrate being associated exclusively with the MSD1 insert in human skeletal muscle (Walsh et al., 1989). It is likely that additional N- and O-linked oligosaccharides will be discovered on N-CAM, either directly on the proteins or associated with one of the alternatively spliced inserts such as MSD1.

Glycosylation of N-CAM may be involved in regulating the amount of N-CAM present at the cell surface, altering the ligand properties of N-CAM, and modulating interaction between N-CAM and other cell surface proteins. Although not proven, O-linked glycosylation may be involved in stabilizing N-CAM and increasing its cell surface expression (Walsh et al., 1989). This would be important because small variations in the surface density of N-CAM affect the rate and amount of homophilic binding (Hoffman and Edelman, 1983). Alternatively, O-linked glycosylation might influence or expand the ligand properties of N-CAM, either by modifying N-CAM's homophilic binding or by enhancing N-CAM's capacity to interact with extracellular matrix or other cell surface molecules (Walsh et al., 1989; Kadmon et al., 1990a). In the case of N-linked carbohydrates, there is direct evidence that these moieties are involved in modulating N-CAM's homophilic binding and in altering the function of other proteins that are associated with N-CAM (Hoffman and Edelman, 1983; Cole and Schachner, 1987; Kunemund et al., 1988; Rutishauser et al., 1988; Kadmon et al., 1990a, b). Of these N-linked moieties, the best characterized is polysialic acid (PSA).

PSA is a large, negatively-charged moiety that attaches to the N-CAM proteins and reduces the extent of membrane contact between apposed cells and also between individual cells and their underlying substratum (Rothbard et al., 1982; Finne et al., 1983; Acheson et al., 1991). Removal of PSA leads to an increase in the amount and intimacy of surface-surface contact and thus to enhanced interaction between membrane-associated ligands, one of which is N-CAM itself (see Fig. I-4) (Hoffman and Edelman, 1983; Rutishauser et al., 1985, 1988; Acheson and Rutishauser, 1988; Acheson et al., 1991). For example, removal of PSA increases N-CAM homophilic binding (Hoffman and Edelman, 1983) but also (a) causes a contact-dependent increase in choline acetyltransferase activity in chick sympathetic neurons (Acheson and Rutishauser, 1988), (b) augments adhesion mediated by another cell adhesion molecule, L1 (Ng-CAM), that associates with N-CAM on the cell surface (Kadmon et al., 1990a, b; Acheson et al., 1991) and, (c) increases cell-substrate interaction (Acheson et al., 1991). PSA therefore affects not only the function of N-CAM by decreasing its binding, but also the function of other ligands that may or may not be directly associated with N-CAM-mediated adhesion.

The expression of PSA on N-CAM varies during the development of individual tissues but, at the level of the whole embryo, there is more PSA present on N-CAM at younger stages and less PSA on N-CAM in older embryos (Rothbard et al., 1982; Sunshine et al., 1987). This shift in the amount of PSA is termed E (embryonic) to A (adult) conversion. The larger amount of PSA in early embryos suggests that, at this stage, the PSA may be involved in lowering

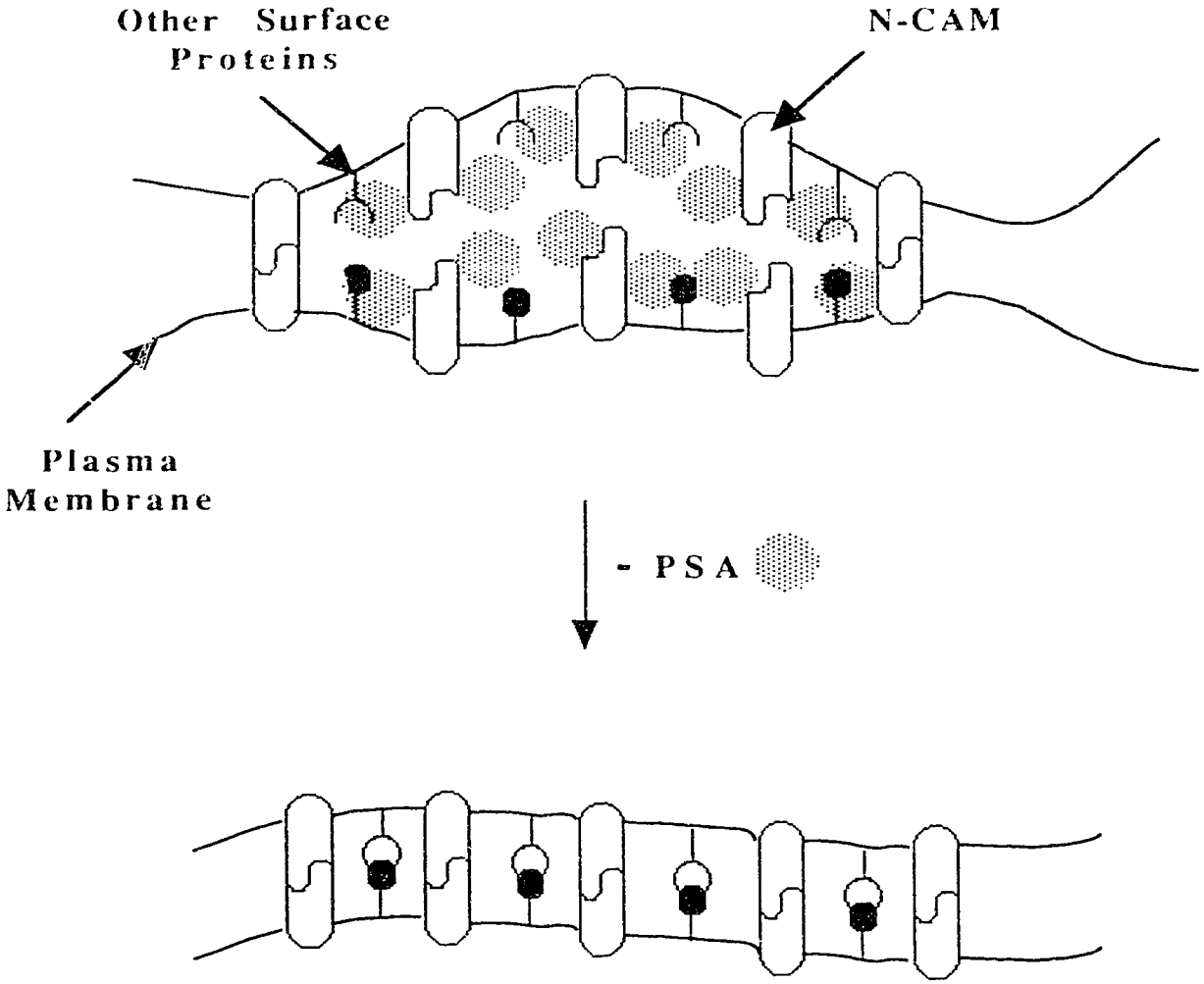


Fig. I-4. Effect of polysialic acid (PSA) on N-CAM homophilic binding and other contact-dependent events. PSA is large and negatively-charged, and prevents contact between the plasma membranes of apposed cells. Removal of PSA increases the amount and intimacy of surface-surface contact and leads to enhanced interaction between membrane-associated ligands, one of which is N-CAM.

(Modified after Rutishauser et al., 1988).

adhesion to allow for cell migration or division (Rothbard et al., 1982; Rutishauser et al., 1985). Decreases in PSA as development proceeds may then be important for increasing N-CAM-mediated adhesion to stabilize tissue architecture later in development (Hoffman and Edelman, 1983). At present, little is known of the specific function of PSA. However, in some instances, it appears that PSA may be more important in regulating cell-cell and cell-extracellular matrix (ECM) interactions than adhesion mediated by the N-CAM polypeptides per se (Acheson et al., 1991).

Role of N-CAM-mediated adhesion in development

N-CAM is thought to be involved in the formation of many embryonic tissues including brain, heart, kidney, muscle, and feather, but little is known about the specific role it plays in the morphogenesis of these structures. Two approaches are generally used to examine the function of N-CAM, namely (a) treating developing tissues with either antibodies that inhibit N-CAM homophilic binding or with an enzyme, endoneuraminidase-N, that selectively removes PSA (Buskirk et al., 1980; Fraser et al., 1984; Keilhauer et al., 1985; Rutishauser et al., 1985; Maier et al., 1986; Remsen et al., 1990; Aoki et al., 1991; Jiang and Chuong, 1992) and, (b) transfecting specific N-CAM proteins into various cell lines in order to examine the function of individual N-CAM polypeptides under particular conditions (Gower et al., 1988; Doherty et al., 1991; Lanier et al., 1991). These experiments show that N-CAM not only holds cells together but also influences a variety of other processes such as cell division (Rutishauser et al., 1985; Aoki et al., 1991), migration

(Rutishauser et al., 1985), death (Nitta et al., 1989; Lanier et al., 1991), fusion (Knudsen et al., 1990), signal transduction (Schuch et al., 1989; Doherty et al., 1991), and interaction with ECM (Cole et al., 1985; Cole and Glaser, 1986; Cole et al., 1986; Probstmeier et al., 1989, 1992; Acheson et al., 1991). It thus appears that N-CAM may have a role in modulating other primary cell functions (division, death, migration) and may be important in directing the initial formation of, and subsequent interactions between, cell populations in the embryo.

(a) Developmental role of N-CAM-mediated adhesion

The idea that cell adhesion molecules (CAMs), such as N-CAM, play a central role in morphogenesis by acting as regulators of other primary cellular processes has been termed the regulator hypothesis (Edelman, 1984a) (see Fig. I-5). During development of the embryo, morphogenetic movements bring appropriate cell populations together in a continuous set of interactions involving both cell contact and other molecular signals. The interactions of these cells at specific times and locations leads to embryonic induction, subsequent differentiation, and eventually to establishment of form. The regulator hypothesis suggests that genes for CAMs are expressed prior to the genes involved in differentiation, allowing CAMs to direct the movement of cell populations within the embryo and determine which cell populations interact with one another. By varying both the expression pattern of CAMs and their adhesive properties during morphogenesis, other primary cell processes such as migration and division may also be altered, allowing cells of different histories to be

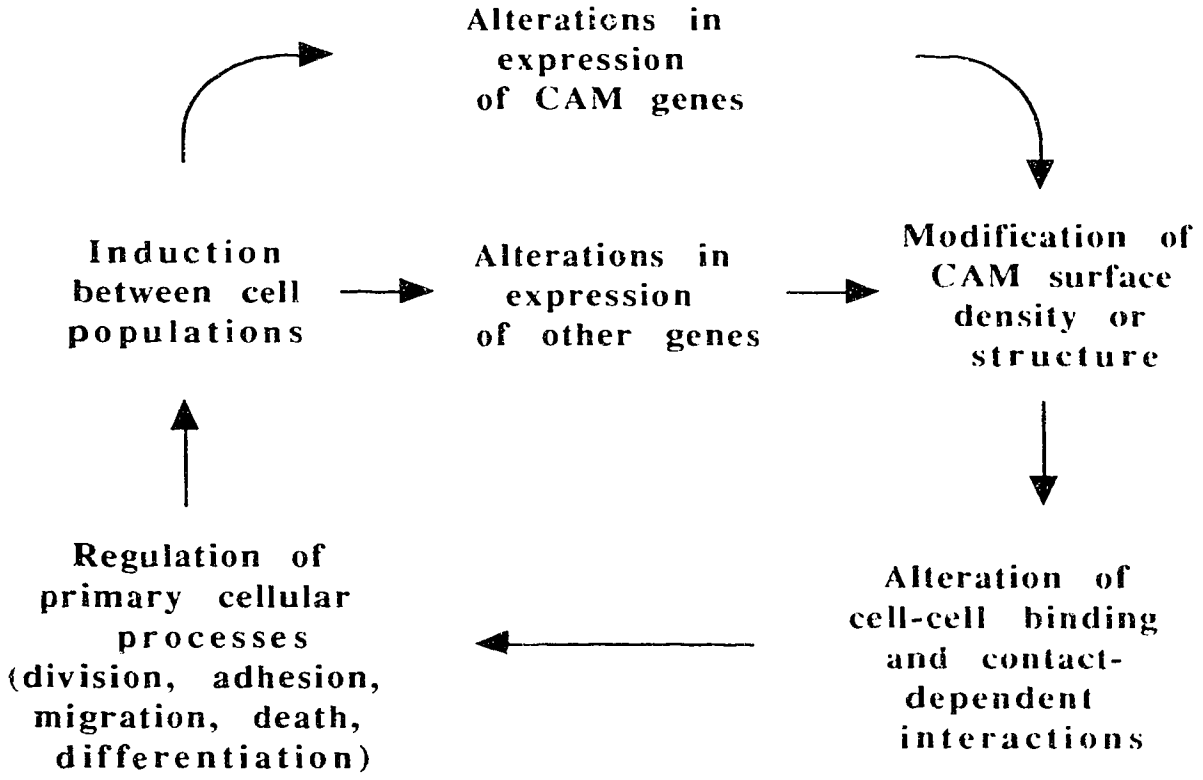


Fig. I-5. Overview of the regulator hypothesis, which suggests that genes for cell adhesion molecules (CAMs) are expressed prior to genes involved in differentiation. By varying cell adhesion during development, contact between cells can be modulated, leading to changes in cell-cell interaction and in the primary cellular processes. By regulating the primary cellular processes, CAMs may be able to determine which cell populations interact during embryogenesis.

(Modified after Edelman, 1984a).

brought together and the proper sequence of interactions to occur. The increasing amount of data produced from perturbation and transfection experiments support this hypothesis by demonstrating that N-CAM-mediated adhesion is indeed interconnected to the other primary cellular processes.

A common theme underlying the studies of N-CAM function is that N-CAM mediates both cell-cell and cell-ECM interaction, these interactions are altered by modifications of the N-CAM proteins (as described above), and perturbation of these interactions causes changes in other cellular activities. Addition of PSA to N-CAM is the best understood modification, PSA reducing the extent of membrane contact between apposed cells and also between individual cells and ECM (Rutishauser et al., 1988; Acheson et al., 1991). PSA thus decreases N-CAM homophilic binding due to reduced membrane-membrane interaction (Hoffman and Edelman, 1983) but also reduces interaction between other cell surface molecules and their specific cellular or extracellular ligands (Acheson and Rutishauser, 1988; Kadmon et al., 1990a, b; Acheson et al., 1991). Keeping in mind the idea that N-CAM mediates cellular interactions and that PSA decreases them, the changes that occur upon perturbing N-CAM-mediated adhesion can be explained. For example, N-CAM mediates adhesion between fasciculating neurons in brain, and between fusing myoblast cells in muscle (Rutishauser et al., 1978; Knudsen et al., 1990). Removal of PSA from N-CAM in these tissues increases N-CAM binding and enhances neuron fasciculation and myoblast fusion, whereas antibodies to N-CAM reverse this enhancement and interfere with these events (Keilhauer et al., 1985; Knudsen et al.,

1990). Similarly, removal of PSA from N-CAM increases cell-ECM interaction, but antibodies to N-CAM interfere with this process (Cole and Glaser, 1986; Acheson et al., 1991). The decreases in cell-cell and cell-ECM interaction caused by perturbing N-CAM also affect other contact-dependent interactions and lead to additional changes in cell division, migration, death, and differentiation. For example, perturbation of normal cell-ECM interaction of fibroblastic cells with anti-N-CAM antibodies causes large increases in cell division (Aoki et al., 1991). Likewise, treatment of developing retina with anti-N-CAM antibodies and with endo-N causes changes in morphology and histology by apparently altering normal cell division and migration (Buskirk et al., 1980; Rutishauser et al., 1985). These experiments thus support the idea that N-CAM may have a regulatory role in development and may play a central role in modeling embryonic form.

(b) Developmental roles of individual N-CAM isoforms

Although the developmental function of N-CAM-mediated adhesion as a whole is becoming clearer, the roles of the individual N-CAM polypeptides remain unclear. As mentioned above, the N-CAM proteins differ in their interaction with the cell membrane; the two largest proteins are transmembrane, whereas the smallest protein is attached to the membrane via a lipid anchor. Due to these differences in structure, the proteins interact differently with the cytoskeleton and also have different mobilities within the cell membrane (Pollerberg et al., 1985, 1986). Since cell migration and sorting probably depend upon a variety of factors such as the surface

concentration of CAMs (Thiery et al., 1982), CAM flexibility at regions where the cell membrane is changing shape (Prediger et al., 1988; Knudsen et al., 1989), and interaction of CAMs with cytoskeletal components (Jaffe et al., 1990), selective expression of individual N-CAM proteins may be responsible for changing these parameters and for regulating migration and sorting. In particular, the 180 kD N-CAM protein interacts with the cytoskeleton (Pollerberg et al., 1987) and is expressed late in development, suggesting it may play a role in stabilization of tissue architecture (Pollerberg et al., 1985). Likewise, the lipid-linked 120 kD N-CAM protein tends to be expressed on cells undergoing lateral motions such as in skeletal muscle and heart (Knudsen et al., 1989; Wharton et al., 1989), perhaps due to its greater mobility and flexibility within the cell membrane (Pollerberg et al., 1986). In polarized epithelial cells, the N-CAM proteins are selectively targeted to the apical and basolateral membrane domains due to their differences in structure, suggesting an additional function of the different proteins could be to specify their own cellular localization and thus control site-specific cell-cell or cell-ECM interaction (Powell et al., 1991). Additional experiments remain to be done in order to verify these ideas and to clarify the roles of the individual N-CAM proteins in morphogenesis.

To gain insight into the function of the different proteins, it will be useful to determine which of the N-CAM molecules are restricted to particular tissues or to particular stages of development. In this way, correlations between protein expression and morphogenetic events can be established, and additional clues about protein function can be obtained. To this end, a useful system in which to

examine the expression of N-CAM during morphogenesis is feather formation. Feather development is a striking example of embryonic induction leading to the formation and patterning of multiple, discrete cell populations within a tissue. N-CAM is known to be expressed in a specific spatiotemporal pattern in developing feathers (Chuong and Edelman, 1985a, b), suggesting that N-CAM-mediated adhesion may play a role in feather morphogenesis. However, it is not known what forms of N-CAM are expressed in feathers, and little is known about the effects of perturbing N-CAM-mediated adhesion in this system. As such, feathers offer a model in which the N-CAM proteins can be characterized and their potential role in formation and patterning of cell populations examined.

Expression pattern of N-CAM in developing feathers

Chicken skin consists of two cell layers, an epidermis of ectodermal origin and dermis of mesodermal origin. Both the initial formation and subsequent development of feathers depend upon interactions between these two tissue layers; if the two layers are separated prior to the onset of feather formation, neither one will develop (Rawles, 1963). The epidermis is thought to initiate feather formation by inducing changes in the dermis, but the pattern and type of appendage formed in the skin is specified by the dermis (Sengel and Abbott, 1960; Goetinck and Abott, 1963; Rawles, 1963; Wessells, 1965; Dhouailly and Sawyer, 1984). During later stages of feather formation, continued interaction between epidermis and dermis is required for proper development of the feather filaments

(Sawyer and Fallon, 1986). Thus, by interacting with one another, the two tissue layers generate a pattern that neither tissue alone is capable of producing.

Feather development can be separated into two major phases consisting of (a) formation and patterning of the feather primordia or rudiments (embryonic days 7-9, E7-E9) and, (b) elongation of the rudiments into filaments and development of mature feathers (E10-E20) (see Fig. I-6). During the course of feather formation, N-CAM is expressed on cells involved in the induction and formation of the feather rudiments, cells that divide to form the elongating filaments, and on cells that are thought to die to create the branches of the mature feather (Chuong and Edelman, 1985a). It is therefore reasonable to conjecture that N-CAM-mediated adhesion may be involved in regulating these events and may have a role in directing feather formation. The following description of feather development is for the dorsal skin of the chicken, which is one of the earliest feather tracts to develop (Lucas and Stettenheim, 1972) and is the easiest to manipulate.

(a) Formation and patterning of the feather rudiments

Prior to the initiation of feather formation (E6), the dermis consists of a loose population of cells and is overlaid by a uniform layer of epidermal cells. No feather rudiments are present (Fig. I-6). At this stage, N-CAM is expressed at low levels on cells of dermis (Chuong and Edelman, 1985a).

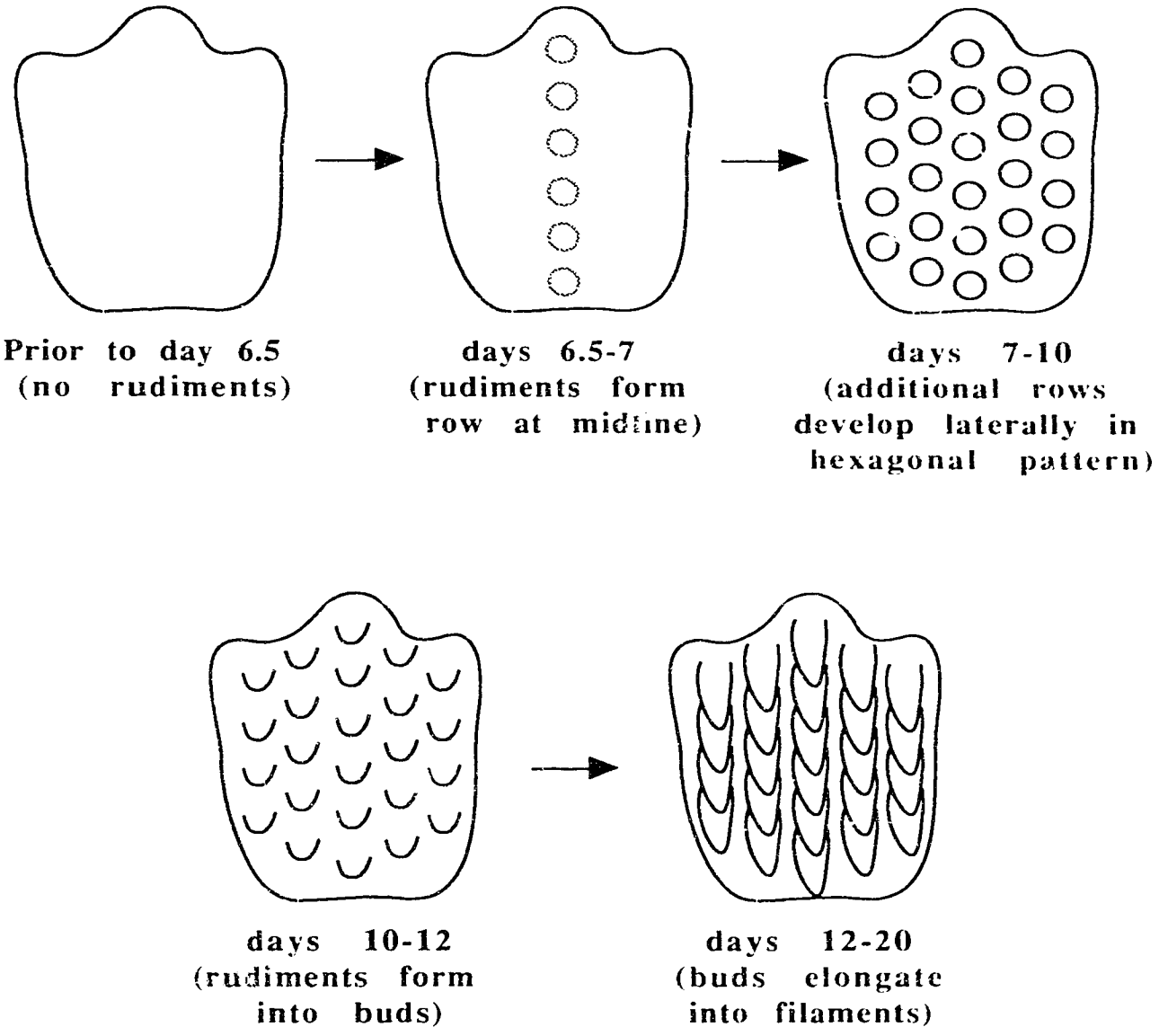


Fig. I-6. Schematic of feather formation in dorsal embryonic chicken skin, showing the time course of development.

Between E6 and E7, the epidermis induces the dermal cells to aggregate (Rawles, 1963; Sengel, 1976), and individual feather rudiments start to form at the midline of the skin (Fig. I-6). Each rudiment consists of a condensed population of dermal cells (dermal condensation) overlaid by a thickening in the epidermis (epidermal placode) (Wessells, 1965; Lucas and Stettenheim, 1972). From E7 to E9, subsequent rows of rudiments develop in a bilateral direction from the midline of the skin and are laid down in a hexagonal pattern (Davidson, 1983a, b; Mayerson and Fallon, 1985) (Fig. I-6). During this period the expression of N-CAM changes; N-CAM that was initially present at low levels throughout the dermis now becomes strongly expressed on cells that are forming the dermal condensations and decreases in expression in areas of skin that will not form feathers (Chuong and Edelman, 1985a). Within each feather rudiment the distribution of N-CAM becomes polarized and N-CAM is expressed ma in the cephalic region of the dermal condensation. This is the region of the condensation that undergoes the least cell division as the rudiment first begins to elongate into a filament (Wessells, 1965; Desbien et al., 1991). It is therefore possible that the increased amount of N-CAM in the cephalic region may be involved in the decreased cell division in this area.

The feather rudiments now start to push above the surface of the skin due to an increase in cell division within them. Just prior to the onset of this outgrowth (E9-E10), there is a strong expression of N-CAM on cells of the epidermal placode above the center of the N-CAM-rich dermal condensation (Chuong and Edelman, 1985a). This expression of N-CAM coincides with a known inductive signal

sent from the epidermis to the dermis to begin filament formation (Lucas and Stettenheim, 1972; Sengel, 1976). If epidermis and dermis are separated at this stage, filament formation does not occur, suggesting that direct contact between the two tissue layers is required for filament outgrowth (Konig and Sawyer, 1985). It is possible that the transient, increased expression of N-CAM on both the dermal and epidermal cells may allow for direct cell-cell contact and for the exchange of signals between the two tissue layers at this time.

(b) Elongation of the rudiments into filaments and formation of mature feathers

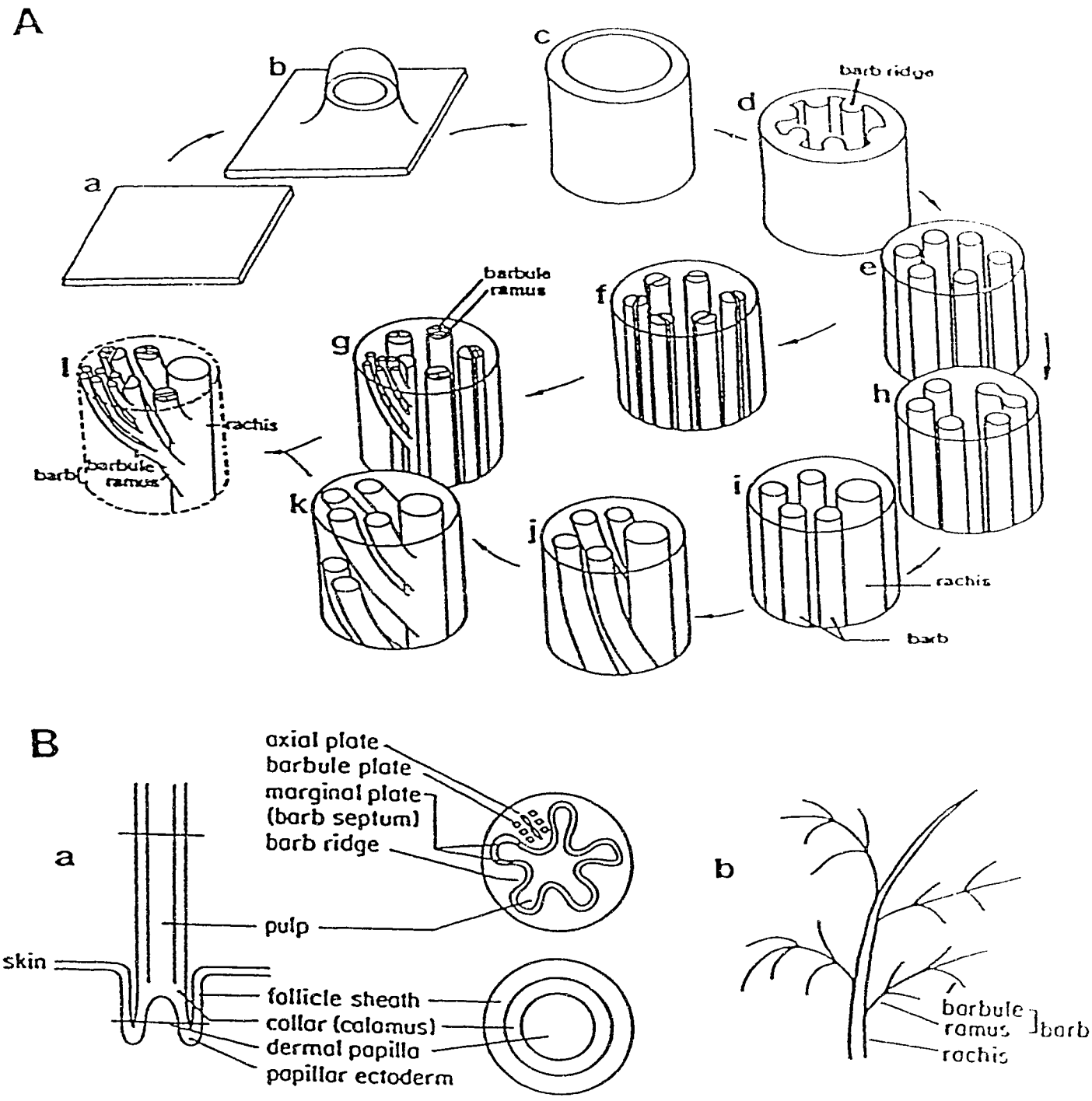
As elongation of the feather rudiments proceeds, the epidermis immediately surrounding the rudiments begins to invaginate to form a double-layered follicle (E10-E11) (Lucas and Stettenheim, 1972). The dermal condensation of the rudiment becomes encased by the walls of the ingrowing follicle and forms a dermal papilla from which the central portion (pulp, blood vessels, nerves) of the filament will arise (see Fig. I-7). Around the papilla, the epidermis thickens due to active cell division and forms a ring, called the epidermal collar, from which the inner layer of the follicle is derived. The follicle consists of a single outer layer that will form a sheath around the feather, and of an inner layer that is separated into (i) an external intermediate layer and (ii) a central basilar layer (Fig. I-7). The intermediate layer will become the major building blocks of the feather (barbs and barbules), whereas the basilar layer will form a single cell layer that separates the intermediate layer from the core

Fig. I-7. Schematic representation of filament formation and differentiation into a mature feather.

(Part A) Illustration showing how a flat layer of skin is transformed into the structure of a mature feather. Note that the number and sizes of each structure are only representative and do not reflect the actual values. (a) Flat sheet of skin. (b) Formation of feather bud. (c) A segment of the elongated bud, showing formation of an epithelial cylinder. (d and e) Invagination and cleavage of the epithelial layer into many barb ridges, which later become the individual barbs. (f and g) Segregation of each barb ridge into ramus and barbule plates. (h and i) Fusion of barb ridges into the larger rachis. (j and k) Insertion of the barbs onto the rachis. (l) A combination of processes e to g and processes e to k forms a feather structure composed of three levels of branching: tertiary branches (barbules) onto secondary branches (ramus), and secondary branches onto a primary branch (rachis).

(Part B) Feather structures are shown with appropriate terminology. (a) Longitudinal section of a developing filament. Cross sections at the levels of the two lines indicated on the longitudinal secton are shown to the right. (b) Structure of a bilaterally symmetrical adult feather.

(Taken from Chuong and Edelman, 1985a).


A
a
b
c
d
barb ridge
e
f
g
barbule
ramus
h
i
j
k
l
rachis
barb
barbule
ramus
rachis
barb
B
a
axial plate
barbule plate
marginal plate
(barb septum)
barb ridge
pulp
skin
follicle sheath
collar (calamus)
dermal papilla
papillar ectoderm
b
barbule
ramus
barb
rachis

of dermal cells (pulp). At the base of each follicle, erector muscles are becoming organized in the surrounding dermis. These muscles may reach the wall of the follicle but do not attach to it at this time. Throughout this developmental period, N-CAM is expressed on cells that will form the dermal papilla, epidermal collar, and the muscles of the feather (Chuong and Edelman, 1985a).

Barb ridges now begin to arise from the intermediate layer of the follicle by rearrangement of cells into a series of ridges that are parallel to the long axis of the filament (Lucas and Stettenheim, 1972) (Fig. I-7). Nine to eleven ridges develop around the circumference and lengthen towards the base of the filament. The internal basilar layer follows the contours of these ridges and forms into marginal plates (barb septa). Within each barb ridge, the cells reorganize into two rows called barbule plates (Fig. I-7). The barbule plates are separated from one another by a layer of cells called the axial plate. The cells of the barbule plates will form two structures: the cells closest to the central pulp will become a major branch of the feather (ramus), whereas the more lateral cells will become minor branches (barbules). Cells of the axial plate degenerate to form two rows of barbules, which subsequently attach onto the central ramus (Fig. I-7). Cells of the marginal plate also degenerate to create individual barbs, each barb containing a ramus and the two rows of barbules. Several of the barbs fuse to form the main stem (rachis) of the feather. The remaining barbs attach to the rachis in a similar fashion to which the rows of barbules attached to the ramus. In this manner the final branched structure of the feather is formed. During this process (E12-E16), N-CAM is expressed on the cells of the axial

and marginal plates that degenerate to create the spaces between the barbules and the barbs (Chuong and Edelman, 1985a). It is thought that these cells may undergo programmed death, suggesting that the expression of N-CAM on these cells may be involved in determining their fate and thus in creating the final structure of the feather.

The remainder of feather development (E16-E20) consists of additional elongation of the filament, resorption of the pulp, and keratinization of the barbs (Lucas and Stettenheim, 1972; Haake et al., 1984). The filament becomes primarily acellular, except at its base, where N-CAM continues to be expressed on cells of the dermal papilla, epidermal collar, and the muscle cells around the follicle. Shortly after hatching, the sheath around each filament dries and flakes away and the individual barbs are released (Lucas and Stettenheim, 1972). As well, the erector muscles attach to the base of each filament to allow for the movement of the individual feathers (Lucas and Stettenheim, 1972).

Experimental Objectives

When the expression pattern of N-CAM in developing feathers is placed into the context of the regulator hypothesis, it is clear that N-CAM-mediated adhesion may be involved in directing feather morphogenesis by regulating epidermal-dermal interaction, cell division, and perhaps even cell death. At present, neither the forms of the N-CAM polypeptides nor the expression pattern of PSA have been examined in detail in developing feathers. As well, only preliminary investigations into the function of N-CAM-mediated adhesion in developing feathers have been done (Jiang and Chuong,

1992). The goals of this thesis were therefore to (a) characterize the various molecular forms of N-CAM present in developing feathers and (b) determine in greater detail what changes in feather development occur upon perturbing N-CAM-mediated adhesion in this system. By correlating changes in morphogenesis with perturbation of N-CAM-mediated adhesion, information about the role of N-CAM in developing feathers should be obtained.

References

Acheson, A. and Rutishauser, U. (1988). Neural cell adhesion molecule regulates cell contact-mediated changes in choline acetyltransferase activity of embryonic chick sympathetic neurons. *J. Cell Biol.* **106**, 479-486.

Acheson, A., Sunshine, J. L. and Rutishauser, U. (1991). N-CAM polysialic acid can regulate both cell-cell and cell-substrate interactions. *J. Cell Biol.* **114**, 143-153.

Aoki, J., Umeda, M., Takio, K., Titani, K., Utsumi, H., Sasaki, M. and Inoue, K. (1991). Neural cell adhesion molecule mediates contact-dependent inhibition of growth of near-diploid mouse fibroblast cell line m5S/1M. *J. Cell Biol.* **115**, 1751-1761.

Barthels, D., Santoni, M.-J., Wille, W., Ruppert, C., Chaix, J.-C., Hirsch, M.-R., Fontecilla-Camps, J. C. and Goridis, C. (1987). Isolation and nucleotide sequence of mouse N-CAM cDNA that codes for a Mr 79000 polypeptide without a membrane-spanning region. *EMBO J.* **6**, 907-914.

Barton, C. H., Dickson, G., Gower, H. J., Rowett, L. H., Putt, W., Elsom, V., Moore, S. E., Goridis, C. and Walsh, F. S. (1988). Complete sequence and in vitro expression of a tissue-specific phosphatidylinositol-linked N-CAM isoform from skeletal muscle. *Development* **104**, 165-173.

Bertolotti, R., Rutishauser, U. and Edelman, G. M. (1980). A cell surface molecule involved in aggregation of embryonic liver cells. *Proc. Natl. Acad. Sci. USA* **77**, 4831-4835.

Brackenbury, R., Thiery, J.-P., Rutishauser, U. and Edelman, G. M. (1977). Adhesion among neural cells of the chick embryo. I. An immunological assay for molecules involved in cell-cell binding. *J. Biol. Chem.* **252**, 6835-6840.

Buskirk, D. R., Thiery, J.-P., Rutishauser, U. and Edelman, G. M. (1980). Antibodies to a neural cell adhesion molecule disrupt histogenesis in cultured chick retinae. *Nature* **285**, 488-489.

Chen, A., Reyes, A. and Akeson, R. (1990). Transcription initiation sites and structural organization of the extreme 5' region of the rat neural cell adhesion molecule gene. *Mol. Cell. Biol.* **10**, 3314-3324.

Chuong, C.-M. and Edelman, G. M. (1984). Alterations in neural cell adhesion molecules during development of different regions of the nervous system. *J. Neurosci.* **4**, 2354-2368.

Chuong, C.-M. and Edelman, G. M. (1985a). Expression of cell-adhesion molecules in embryonic induction. I. Morphogenesis of nestling feathers. *J. Cell Biol.* **101**, 1009-1026.

Chuong, C.-M. and Edelman, G. M. (1985b). Expression of cell-adhesion molecules in embryonic induction. II. Morphogenesis of adult feathers. *J. Cell Biol.* **101**, 1027-1043.

Cole, G. J. and Glaser, L. (1986). A heparin-binding domain from N-CAM is involved in neural cell-substratum adhesion. *J. Cell Biol.* **102**, 403-412.

Cole, G. J., Loewy, A. and Glaser, L. (1986). Neuronal cell-cell adhesion depends on interactions of N-CAM with heparin-like molecules. *Nature* **320**, 445-447.

Cole, G. J. and Schachner, M. (1987). Localization of the L2 monoclonal antibody binding site on chicken neural cell adhesion molecule (N-CAM) and evidence for its role in N-CAM-mediated adhesion. *Neurosci. Let.* **78**, 227-232.

Cole, G. J., Schubert, D. and Glaser, L. (1985). Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions. *J. Cell Biol.* **100**, 1192-1199.

Colewell, G., Li, B., Brady-Kalnay, S. and Brackenbury, R. (1991). Control of N-CAM expression in normal and RSV-transformed cells. *J. Cell Biol.* **115**, 69a.

Covault, J., Merlie, J. P., Goridis, C. and Sanes, J. R. (1986). Molecular forms of N-CAM and its RNA in developing and denervated skeletal muscle. *J. Cell Biol.* **102**, 731-739.

Crossin, K. L., Chuong, C.-M. and Edelman, G. M. (1985). Expression sequences of cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* **82**, 6942-6951.

Crossin, K. L., Edelman, G. M. and Cunningham, B. A. (1984). Mapping of three carbohydrate attachment sites in embryonic and adult forms of the neural cell adhesion molecule. *J. Cell Biol.* **99**, 1848-1855.

Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R. and Edelman, G. M. (1987). Neural cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* **236**, 799-806.

Cunningham, B. A., Hoffman, S., Rutishauser, U., Hemperly, J. J. and Edelman, G. M. (1983). Molecular topography of the neural cell adhesion molecule N-CAM: Surface orientation and location of sialic acid-rich and binding regions. *Proc. Natl. Acad. Sci. USA* **80**, 3116-3120.

Daniloff, J. K., Chuong, C.-M., Levi, G. and Edelman, G. M. (1986). Differential distribution of cell adhesion molecules during histogenesis of the chick nervous system. *J. Neurosci.* **6**, 739-757.

Davidson, D. (1983a). The mechanism of feather pattern development in the chick. I. The time of determination of feather position. *J. Embryol. Exp. Morphol.* **74**, 245-259.

Davidson, D. (1983b). The mechanism of feather pattern development in the chick. II. Control of the sequence of pattern formation. *J. Embryol. Exp. Morphol.* **74**, 261-273.

Desbien, X., Queva, C., Jaffredo, T., Stehelin, D. and Vandenbunder, B. (1991). The relationship between cell proliferation and the transcription of the nuclear oncogenes c-myc, c-myb, c-ets-1 during feather morphogenesis in the chick embryo. *Development* **111**, 699-713.

Dickson, G., Gower, H. J., Barton, C. H., Prentice, H. M., Elsom, V. L., Moore, S. E., Cox, R. D., Quinn, C., Putt, W. and Walsh, F. S. (1987). Human muscle neural cell adhesion molecule (N-CAM): Identification of a muscle-specific sequence in the extracellular domain. *Cell* **60**, 1119-1130.

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

Edelman, G. M. (1984a). Cell adhesion and morphogenesis: The regulator hypothesis. *Proc. Natl. Acad. Sci. USA* **81**, 1460-1464.

Edelman, G. M., Gallin, W. J., Delouvee, A., Cunningham, B. A. and Thiery, J.-P. (1983). Early epochal maps of two different cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* **80**, 4384-4388.

Finne, J., Finne, U., Deagostini-Bazin, H. and Goridis, C. (1983). Occurrence of α2-8 linked polysialosyl units in a neural cell adhesion molecule. *Biochem. Biophys. Res. Commun.* **112**, 482-487.

Fraser, S. E., Murray, B. A., Chuong, C.-M. and Edelman, G. M. (1984). Alteration of the retinotectal map in *Xenopus* by antibodies to neural cell adhesion molecules. *Proc. Natl. Acad. Sci. USA* **81**, 4222-4226.

Gallin, W. J., Edelman, G. M. and Cunningham, B. A. (1983). Characterization of L-CAM, a major cell adhesion molecule from embryonic liver cells. *Proc. Natl. Acad. Sci. USA* **80**, 1038-1042.

Galtsoff, P. S. (1925). Regeneration after dissociation (an experimental study on sponges). II. Histogenesis of *Microciona prolifera* Verr. *J. Exp. Zool.* **42**, 223-251.

Gennarini, G., Rougon, G., Deagostini-Bazin, H., Hirn, M. and Goridis, C. (1984). Studies on the transmembrane disposition of the neural cell adhesion molecule N-CAM. *Europ. J. Biol.* **42**, 1-8.

Goridis, C. and Wille, W. (1988). The three size classes of mouse N-CAM proteins arise from a single gene by a combination of alternative splicing and use of different polyadenylation signals. *Neurochem. Int.* **12**, 269-272.

Gower, H. J., Barton, C. H., Elsom, V. L., Thompson, J., Moore, S. E., Dickson, G. and Walsh, F. S. (1988). Alternative splicing generates a secreted form of N-CAM in muscle and brain. *Cell* **55**, 955-964.

Grumet, M. and Edelman, G. M. (1984). Heterotypic binding between neuronal membrane vesicles and glial cells is mediated by a specific cell adhesion molecule. *J. Cell Biol.* **98**, 1746-1756.

Grumet, M., Hoffman, S. and Edelman, G. M. (1984a). Two antigenically related neuronal cell adhesion molecules of different specifities mediate neuron-neuron and neuron-glia adhesion. *Proc. Natl. Acad. Sci. USA* **81**, 267-271.

Hall, A. K., Nelson, R. and Rutishauser, U. (1990). Binding properties of detergent-solubilized N-CAM. *J. Cell Biol.* **110**, 817-824.

He, H.-T., Finne, J. and Goridis, C. (1987). Biosynthesis, membrane association, and release of N-CAM-120, a phosphotidylinositol-linked form of the neural cell adhesion molecule. *J. Cell Biol.* **105**, 2489-2500.

Hemperly, J. J., Edelman, G. M. and Cunningham, B. A. (1986b). cDNA clones of the neural cell adhesion molecule (N-CAM) lacking a membrane-spanning region consistent with evidence for membrane attachment via a phosphatidylinositol intermediate. *Proc. Natl. Acad. Sci. USA* **83**, 9822-9826.

Hemperly, J. J., Murray, B. A., Edelman, G. M. and Cunningham, B. A. (1986a). Sequence of a cDNA clone encoding the polysialic acid-rich and cytoplasmic domains of the neural cell adhesion molecule N-CAM. *Proc. Natl. Acad. Sci. USA* **83**, 3037-3041.

Hirsch, M.-R., Gaugler, L., Deagostini-Bazin, H., Bally-Cuif, L. and Goridis, C. (1990). Identification of positive and negative regulatory elements governing cell-type-specific expression of the neural cell adhesion molecule gene. *Molec. Cell. Biol.* **10**, 1959-1968.

Hoffman, S. and Edelman, G. M. (1983). Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc. Natl. Acad. Sci. USA* **80**, 5762-5766.

Hoffman, S., Sorkin, B. C., White, P. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B. A. and Edelman, G. M. (1982). Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J. Biol. Chem.* **257**, 7720-7729.

Jaffe, S. H., Friedlander, D. R., Matsuzaki, F., Crossin, K. L., Cunningham, B. A. and Edelman, G. M. (1990). Differential effects of cytoplasmic domains of cell adhesion molecules on cell aggregation and sorting-out. *Proc. Natl. Acad. Sci. USA* **87**, 3589-3593.

Jiang, T.-X. and Chuong, C.-M. (1992). Mechanism of skin morphogenesis. I. Analyses with antibodies to adhesion molecules tenascin, N-CAM, and integrin. *Dev. Biol.* **150**, 82-98.

Kadmon, G., Kowitz, A., Altevogt, P. and Schachner, M. (1990a). The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions. *J. Cell Biol.* **110**, 193-208.

Kadmon, G., Kowitz, A., Altevogt, P. and Schachner, M. (1990b). Functional cooperation between the neural cell adhesion molecules L1 and N-CAM is carbohydrate dependent. *J. Cell Biol.* **110**, 209-218.

Keilhauer, G., Faissner, A. and Schachner, M. (1985). Differential inhibition of neurone-neurone, neurone-astrocyte and astrocyte-astrocyte adhesion by L1, L2 and N-CAM antibodies. *Nature* **316**, 728-730.

Key, B. and Akeson, R. A. (1990). Olfactory neurons express a unique glycosylated form of the neural cell adhesion molecule (N-CAM). *J. Cell Biol.* **110**, 1729-1743.

Key, B. and Akeson, R. A. (1991). Delineation of olfactory pathways in the frog nervous system by unique glycoconjugates and N-CAM glycoforms. *Neuron* **6**, 381-396.

Knudsen, K. A., McElwee, S. A. and Myers, L. (1990). A role for the neural cell adhesion molecule, N-CAM, in myoblast interaction during myogenesis. *Dev. Biol.* **138**, 159-168.

Knudsen, K. A., Smith, L. and McElwee, S. (1989). Involvement of cell surface phosphatidylinositol-anchored glycoproteins in cell-cell adhesion of chick embryo myoblasts. *J. Cell Biol.* **109**, 1779-1786.

Konig, G. and Sawyer, R. H. (1985). Analysis of morphogenesis and keratinization in transfilter recombinants of feather forming skin. *Dev. Biol.* **109**, 381-392.

Kruse, J., Mailhammer, R., Wernecke, H., Faissner, A., Sommer, I., Goridis, C. and Schachner, M. (1984). Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. *Nature* **311**, 153-155.

Kunemund, V., Jungalwala, F. B., Gunther, F., Chou, D. K. H., Keilhauer, G. and Schachner, M. (1988). The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. *J. Cell Biol.* **106**, 213-223.

Lanier, L. L., Chang, C., Azuma, M., Ruitenberg, J. J., Hemperly [illegible] and Phillips, J. H. (1991). Molecular and functional analysis of human natural killer cell-associated neural cell adhesion molecule (N-CAM/CD56). *J. Immunol.* **146**, 4421-44[illegible]

Lucas, A. M. and Stettenheim, P. R. (1972). Avian anatomy. Integument. Part I and II. Washington, DC., Agricultural Research Service, U.S. Department of Agriculture.

Lyles, J. M., Linnemann, D. and Bock, E. (1984). Biosynthesis of the D2-cell adhesion molecule: Post-translational modifications, intracellular transport, and developmental changes. *J. Cell Biol.* **99**, 2082-2091.

Maier, C. E., Watanabe, M., Singer, M., McQuarrie, I. G., Sunshine, J. and Rutishauser, U. (1986). Expression and function of neural cell adhesion molecule during limb regeneration. *Proc. Natl. Acad. Sci. U.S.A* **83**, 8395-8399.

Mayerson, P. L. and Fallon, J. F. (1985). The spatial and temporal sequence in which feather germs arise in the white leghorn chick embryo. *Dev. Biol.* **109**, 259-267.

Moore, S. E., Thompson, J., Kirkness, V., Dickson, J. G. and Walsh, F. S. (1987). Skeletal muscle neural cell adhesion molecule (N-CAM): Changes in protein and mRNA species during myogenesis of muscle cell lines. *J. Cell Biol.* **105**, 1377-1386.

Moscona, A. (1961). Rotation-mediated histogenetic aggregation of dissociated cells. A quantifiable approach to cell interactions in vitro. *Exp. Cell Res.* **22**, 455-475.

Murray, B. A., Hemperly, J. J., Prediger, E. A., Edelman, G. M. and Cunningham, B. A. (1986). Alternatively spliced mRNAs code for different polypeptide chains of the chicken neural cell adhesion molecule (N-CAM). *J. Cell Biol.* **102**, 189-193.

Nitta, T., Yagita, H., Sato, K. and Okumura, K. (1989). Involvement of CD56 (NKH-1/Leu-19 antigen) in an adhesion molecule in natural killer-target cell interaction. *J. Exp. Med.* **170**, 1757-1761.

Nybroe, O., Linnemann, D. and Bock, E. (1988). N-CAM biosynthesis in brain. *Neurochem. Int.* **12**, 251-262.

Pollerberg, E. G., Sadoul, R., Goridis, C. and Schachner, M. (1985). Selective expression of the 180-kD component of the neural cell adhesion molecule N-CAM during development. *J. Cell Biol.* **101**, 1921-1929.

Pollerberg, G. E., Burridge, K., Krebs, K. E., Goodman, S. R. and Schachner, M. (1987). The 180-kD component of the neural cell adhesion molecule N-CAM is involved in cell-cell contacts and cytoskeleton-membrane interactions. *Cell Tissue Res.* **250**, 227-236.

Pollerberg, G. E., Schachner, M. and Davoust, J. (1986). Differentiation state-dependent surface mobilities of two forms of the neural cell adhesion molecule. *Nature* **324**, 462-465.

Powell, S. K., Cunningham, B. A., Edelman, G. M. and Rodriguez-Boulan, E. (1991). Targeting of transmembrane and GPI-anchored forms of N-CAM to opposite domains of a polarized epithelial cell. *Nature* **353**, 76-77.

Prediger, E. A., Hoffman, S., Edelman, G. M. and Cunningham, B. A. (1988). Four exons encode a 93-base-pair insert in three neural cell adhesion molecule mRNAs specific for chicken heart and skeletal muscle. *Proc. Natl. Acad. Sci. USA* **85**, 9616-9620.

Prieto, A. L., Crossin, K. L., Cunningham, B. A. and Edelman, G. M. (1989). Localization of mRNA for neural cell adhesion molecule (N-CAM) polypeptides in neural and non-neural tissues by in situ hybridization. *Proc. Natl. Acad. Sci. USA* **86**, 9579-9583.

Probstmeier, R., Fahrig, T., Spiess, E. and Schachner, M. (1992). Interactions of the neural cell adhesion molecule and the myelin-associated glycoprotein with collagen type I: Involvement in fibrillogenesis. *J. Cell Biol.* **116**, 1063-1070.

Probstmeier, R., Kuhn, K. and Schachner, M. (1989). Binding properties of the neural cell adhesion molecule to different components of the extracellular matrix. *J. Neurochem.* **53**, 1794-1801.

Rawles, M. E. (1963). Tissue interactions in scale and feather development as studied in dermal-epidermal recombinants. *J. Embryol. Exp. Morphol.* **11**, 765-789.

Remsen, L. G., Strain, G. M., Newman, M. J., Satterlee, N. and Daniloff, J. K. (1990). Antibodies to the neural cell adhesion molecule disrupt functional recovery in injured nerves. *Exp. Neurol.* **110**, 268-273.

Reyes, A. A., Small, S. J. and Akeson, R. (1991). At least 27 alternative spliced forms of the neural cell adhesion molecule mRNA are expressed during rat heart development. *Mol. Cell. Biol.* **11**, 1654-1661.

Rothbard, J. B., Brackenbury, R., Cunningham, B. A. and Edelman, G. M. (1982). Differences in the carbohydrate structures of neural cell adhesion molecules from adult and embryonic chicken brains. *J. Biol. Chem.* **257**, 11064-11069.

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

Rutishauser, U., Gall, W. E. and Edelman, G. M. (1978). Adhesion among neural cells of the chick embryo. IV. Role of the cell surface molecule CAM in the formation of neurite bundles in cultures of spinal ganglia. *J. Cell Biol.* **79**, 382-393.

Rutishauser, U., Watanabe, M., Silver, J., Troy, F. and Vimr, E. R. (1985). Specific alteration of N-CAM-mediated cell adhesion by an endoneuraminidase. *J. Cell Biol.* **101**, 1842-1849.

Sadoul, K., Meyer, A., Low, M. G. and Schachner, M. (1986). Release of the 120 kDa component of the mouse neural cell adhesion molecule N-CAM from cell surfaces by phosphatidylinositol-specific phospholipase C. *Neurosci. Let.* **72**, 341-346.

Santoni, M. J., Barthels, D., Vopper, G., Boned, A., Goridis, C. and Wille, W. (1989). Differential exon usage involving an unusual splicing mechanism generates at least eight types of N-CAM cDNA in mouse brain. *EMBO J.* **8**, 385-391.

Schuch, U., Lohse, M. J. and Schachner, M. (1989). Neural cell adhesion molecules influence second messenger systems. *Neuron* **3**, 13-20.

Sengel, P. (1976). Morphogenesis of Skin. New York, Cambridge University Press.

Small, S. J. and Akeson, R. (1990). Expression of the unique N-CAM VASE exon is independently regulated in distinct tissues during development. *J. Cell Biol.* **111**, 2089-2096.

Small, S. J., Haines, S. L. and Akeson, R. A. (1988). Polypeptide variation in an N-CAM extracellular immunoglobulin-like fold is developmentally regulated through alternative splicing. *Neuron* **1**, 1007-1017.

Sorkin, B. C., Hoffman, S., Edelman, G. M. and Cunningham, B. A. (1984). Sulfation and phosphorylation of the neural cell adhesion molecule, N-CAM. *Science* **225**, 1476-1478.

Steinberg, M. S. (1970). Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of hierarchy among populations of embryonic cells. *J. Exp. Zool.* **173**, 395-433.

Sunshine, J., Balak, K., Rutishauser, U. and Jacobson, M. (1987). Changes in neural cell adhesion molecule (N-CAM) structure during vertebrate neural development. *Proc. Natl. Acad. Sci. USA* **84**, 5986-5990.

Thiery, J.-P., Brackenbury, R., Rutishauser, U. and Edelman, G. M. (1977). Adhesion among neural cells of the chick embryo. *J. Biol. Chem.* **252**, 6841-6845.

Thiery, J.-P., Duband, J.-L., Rutishauser, U. and Edelman, G. M. (1982). Cell adhesion molecules in early chicken embryogenesis. *Proc. Natl. Acad. Sci. USA* **79**, 6737-6741.

Thompson, J., Dickson, G., Moore, S. E., Gower, H. J., Putt, W., Kenimer, J. G., Barton, C. H. and Walsh, F. S. (1989). Alternative splicing of the neural cell adhesion molecule gene generates variant extracellular domain structure in skeletal muscle and brain. *Genes Dev.* **3**, 348-357.

Townes, P. L., and Holtfreter, J. (1955). Directed movements and selective adhesion of embryonic amphibian cells. *J. Exp. Zool.* **128**, 53-120.

Walsh, F. S., Parekh, R. B., Moore, S. E., Dickson, G., Barton, C. H., Gower, H. J., Dwek, R. A. and Rademacher, T. W. (1989). Tissue specific O-linked glycosylation of the neural cell adhesion molecule (N-CAM). *Development* **105**, 803-811.

Wessells, N. K. (1965). Morphology and proliferation during early feather development. *Dev. Biol.* **12**, 131-153.

Wharton, J., Gordon, L., Walsh, F. S., Flanigan, T. P., Moore, S. E. and Polak, J. M. (1989). Neural cell adhesion molecule (N-CAM) expression during cardiac development in the rat. *Brain Research* **483**, 170-176.

Wilson, H. V. (1910). Development of sponges from dissociated tissue cells. *Bull Bur. Fisheries.* **30**,1-30.

Zorn, A. M. and Krieg, P. A. (1992). Developmental regulation of alternative splicing in the mRNA encoding *Xenopus laevis* neural cell adhesion molecule (N-CAM). *Dev. Biol.* **149**, 197-205.

CHAPTER II

Structural Variants of the Neural Cell Adhesion Molecule (N-CAM) in Developing Feathers[1]

Introduction

Cellular adhesion is mediated by proteins called cell adhesion molecules (CAMs), which bind to one another and promote membrane contact between cells (Edelman, 1983). The neural cell adhesion molecule (N-CAM), one of the best characterized CAMs, mediates calcium-independent, homophilic adhesion between many different cell types in the developing embryo (Hoffman et al., 1982; Hall et al., 1990). N-CAM is expressed in specific, regulated patterns in many tissues during embryonic development (Edelman, 1983; Chuong and Edelman, 1985a; Crossin et al., 1985). In many cases, these patterns of expression are coincident with interactive, or inductive, events between cell groups or tissues, for example during kidney development, formation of the nervous system, and formation of the feather (Thiery et al., 1982; Edelman, 1983; Chuong and Edelman, 1985a, b). This implies that control of N-CAM-mediated cellular adhesion may be important in the generation of embryonic form.

There are several ways in which the adhesiveness mediated by N-CAM may be regulated during embryonic development, namely, (a) changes in the amount of N-CAM being expressed (Rutishauser et al., 1978), (b) changes in the structure of N-CAM polypeptides being

---

[1]A version of this chapter has been published. Marsh & Gallin 1992. Developmental Biology. 150: 171-184.

expressed (Pollerberg et al., 1985; Murray et al., 1986) and (c) modification of the N-CAM polypeptides by addition of carbohydrate moieties (Hoffman and Edelman, 1983; Walsh et al., 1989). In embryonic chicken brain, N-CAM exists as at least three protein forms (M_r = 180, 140, and 120 kD) that are encoded by different mRNAs produced from a single gene (Murray et al., 1986). The selective expression of the N-CAM polypeptides on different cell types during development (Pollerberg et al., 1985; Daniloff et al., 1986; Moore et al., 1987) suggests that the different polypeptides may have different functions in morphogenesis. Recently, several additional variants of the N-CAM polypeptides have been identified (Dickson et al., 1987; Prediger et al., 1988; Small et al., 1988; Santoni et al., 1989; Thompson et al., 1989; Small and Akeson, 1990; Reyes et al., 1991; Zorn and Krieg, 1992). These variations, which are due to insertion of small additional peptide sequences within the protein, may serve as attachment sites for carbohydrate moieties that are also involved in tissue-specific regulation of N-CAM-mediated cell-cell adhesion (Walsh et al., 1989).

One carbohydrate moiety that alters the adhesive function of N-CAM is polysialic acid (PSA) (Rothbard et al., 1982; Hoffman and Edelman, 1983; Rutishauser et al., 1988). PSA is large and negatively charged, and it inhibits N-CAM-mediated adhesion by preventing membrane contact between apposed cells (Hoffman and Edelman, 1983; Rutishauser et al., 1988; Acheson et al., 1991). During development of the embryo, the amount of PSA attached to N-CAM varies (Rothbard et al., 1982; Sunshine et al., 1987). Decreases in PSA may be important for increasing N-CAM-mediated adhesion to stabilize

tissue architecture late in development (Hoffman and Edelman, 1983). Alternatively, increased amounts of PSA may be involved in lowering adhesion between cells to allow for cell migration or division (Rothbard et al., 1982; Rutishauser et al., 1985). At present, however, little is known of the specific function of PSA, and of the different N-CAM polypeptides.

Feather development in embryonic chicken offers a model system to examine the function of N-CAM and PSA in the formation and patterning of discrete cell populations within a tissue. During feather development, cells in the dermis proliferate and condense into a defined pattern of cell groups (Davidson, 1983a). These groups, called dermal condensations, are overlain by thickenings in the epidermis called epidermal placodes, which together with the condensations constitute the individual feather rudiments (Davidson, 1983a; Mayerson and Fallon, 1985). Within each rudiment, the dermal and epidermal cells interact, proliferate, and develop into a feather bud (Sengel, 1976; Sawyer and Fallon, 1983). This bud elongates due to cell proliferation, and develops into a feather filament (Wessells, 1965; Lucas and Stettenheim, 1972). Cells within the feather filament rearrange into a series of ridges that are parallel to the long axis of the filament (Lucas and Stettenheim, 1972). These ridges develop into the branches, called barbs and barbules, of the mature feather.

During the development of feathers, N-CAM is expressed on some of the cells that are involved in formation of the feather rudiments and feather filaments (Chuong and Edelman, 1985a). Specifically, N-CAM is expressed on cells that form the dermal condensations,

epidermal cells from which the elongating feather filament arises, and on epidermal cells that are thought to die in the filaments to generate spaces between the barbules. Thus, during feather development, N-CAM is expressed on cells that are actively dividing, and may be expressed on cells undergoing death. It is possible that N-CAM-mediated cellular adhesion may be involved in regulating these events and in directing feather formation. At present, however, the function of N-CAM in developing feathers is unknown.

As a first step towards determining how N-CAM functions in feather development, we have analyzed the structure of N-CAM in the feather. Our results indicate that the function of N-CAM may be modulated by variations in the amount of the protein, variations in the structure of the polypeptide chain, and variations in the N-linked carbohydrates and PSA attached to the N-CAM.

Materials and Methods

Materials - Reagents were purchased from the sources indicated: BioBeads SM-2, rabbit anti-mouse IgM HRP, and materials for SDS-PAGE (Bio-Rad); restriction enzymes (BRL); AMV reverse transcriptase and endoglycosidase-F (Boehringer Mannheim); Mowiol 4-88 (Calbiochem); goat and rabbit serum (Gibco); FITC- and RITC-conjugated secondary antibodies (ICN Immunobiologicals); goat anti-rabbit IgG alkaline phosphatase (Jackson Immunoresearch); Tissue-Tek O.C.T. compound (Miles Scientific); HA Filter (Millipore); 3,3' diaminobenzidine tetrahydrochloride (Polysciences); cesium trifluoroacetate, dNTPs, and Sepharose CL-4B (Pharmacia). All other reagents were obtained from BDH or Sigma.

Fertile White Leghorn eggs were obtained from the University of Alberta Experimental Farm, Poultry Division. Eggs were incubated at 37°C, 55% relative humidity, for up to 18 days. Embryos were staged according to the method of Hamburger and Hamilton (1951). E6 to E18 denote embryonic age from 6- to 18-days after the onset of incubation.

Tris buffered saline (TBS) is 50mM Tris-HCl, 150mM NaCl, pH 8.0. TTBS is TBS, 0.1% Tween 20. Chromogen diluent is TBS, 10mM $MgCl_2$. Phosphate buffered saline (PBS) was made as follows: 8g of NaCl, 0.2g of KCl, 0.2g of KH_2PO_4, 0.15g of Na_2HPO_4/liter (pH 7.4) (Dulbecco and Vogt, 1954).

Antibodies - Rabbit polyclonal and mouse monoclonal antibodies (mAbs) were used as prepared by Hoffman et al. (1982) (anti-N-CAM CAM-6 and 802) and Watanabe et al. (1986) (anti-N-CAM 5e), Dodd et al. (1988) (anti-PSA 5A5), Gallin et al. (1983) (anti-L-CAM 7D6), Lin et al. (1985) (anti-tropomyosin CH1), and Skalli et al. (1986) (anti-α-actin αsm-1). Anti-N-CAM mAbs CAM6 and 5e, and anti-N-CAM polyclonal antibody 802 all react with protein epitopes. All three anti-N-CAM antibodies recognize the 180, 140, and 120 kD polypeptides in embryonic chick brain and do not react with other proteins, indicating that any carbohydrates recognized by anti-N-CAM 802 are unique to N-CAM. All other antibodies listed are monoclonal. An irrelevant mAb against chick liver bile cannaliculi, culture supernatant from SP2/0 myeloma cells, and rabbit or goat serum were used as controls. Anti-α-actin antibody

was purchased from Sigma. Anti-tropomyosin CH1, anti-PSA 5A5, and anti-N-CAM 5e antibodies were from the Developmental Studies Hybridoma Bank (Baltimore, MD).

Immunoperoxidase staining of wholemounts - Skin was removed from stage 29-44 (E6-E18) embryos by blunt dissection. For separation of epidermis and dermis (stage 29-35, E6-E9), skin was mounted epidermal-side down on HA filter, incubated in 2x Tyrode's solution with EDTA (Konig and Sawyer, 1985) for 20 min, and the dermis was peeled off with forceps and mounted on another filter. Separated epidermis and dermis were fixed in 4% paraformaldehyde with 3% hydrogen peroxide overnight at 4°C. Whole-skin samples were fixed on ice for 2 h in a freshly-made solution of 20% dimethylsulfoxide (DMSO) in methanol. Hydrogen peroxide was added to a concentration of 3%, and the sample was left overnight at 4°C. The following day, whole-skin samples were soaked in methanol for several hours to remove excess DMSO. All samples were then washed 2x10 min in TBS and incubated in primary antibody overnight (rabbit antibodies (10 mg/ml) and mouse ascites were diluted 1:900 in 20% goat serum or 20% rabbit serum in TBS, respectively; for mouse mAb culture supernatants, rabbit serum was added to 20% final concentration). Samples were washed 5x1 h in TBS, and then incubated in horseradish peroxidase-conjugated secondary antibody against the appropriate species (secondary antibody was diluted 1:300 in 20% goat or rabbit serum in TBS, accordingly). Samples were washed as above, and the peroxidase was localized using 3,3' diaminobenzidine tetrahydrochloride (DAB) (0.05%) and hydrogen peroxide (0.02%) in TBS. After DAB treatment, samples were washed

several times in TBS, dehydrated through a graded alcohol series, soaked in xylene for several minutes, and mounted on a glass slide with Permount. Wholemounts were photographed on a Zeiss Axiophot microscope using Kodak Tri-X 400 film.

Immunofluorescent staining - Stage 29-35 (E6-E9) embryos and dorsal skins from stage 36-44 (E10-E18) embryos were fixed in 4% paraformaldehyde overnight at 4°C, cryoprotected by immersion in a graded sucrose series, and mounted in Tissue-Tek O.C.T. compound. 10 µm frozen sections were cut on a cryostat, collected on chrome-alum subbed slides, and stored at -80°C. Prior to staining, sections were pre-incubated in 0.7% carrageenan, 0.1% Triton X-100, in TBS (Sofroniew and Schrell, 1982) for 1 h to block non-specific staining. Sections were then incubated overnight with primary antibody (polyclonal antibodies and monoclonal ascites were diluted 1:400 in blocking solution, and mAb culture supernatants were used undiluted). After 5x10 min washes in TBS, sections were incubated with FITC- and RITC-labelled secondary antibodies against the appropriate species (1:200 in carrageenan blocking solution for anti-rabbit antibodies, 1:100 for anti-mouse antibodies) for 2 h, washed five times again, and mounted in Mowiol (Osborn and Webber, 1982), modified by the addition of 2,4-diazabicyclo (2,2,2) octane (Zalik et al., 1990). Sections were photographed on a Zeiss Axiophot microscope using Kodak Tri-X 400 film.

Immunoaffinity Purification of N-CAM - N-CAM was purified using a specific mAb (CAM-6) coupled to Sepharose CL-4B as described previously (Hoffman et al., 1982).

Tissue Extracts, PSA Removal, and Endo-F Digestion - Tissue was homogenized using a Polytron homogenizer (Brinkmann) in 0.5% C[illegible]PS in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 5 µg/ml [illegible]ase, and 1 mM PMSF. Samples were kept on ice for 10 min with occasional vortexing, and cleared by centrifuging 10 min in a microfuge at 4°C. Samples were mixed with SDS-PAGE sample buffer and heated 1 min in a boiling water bath. For PSA removal, samples were heated for 30 min in a boiling water bath or digested with *Clostridium perfringens* neuraminidase as described by Hoffman et al. (1982). For removal of N-linked carbohydrate, desialylated N-CAM was incubated 48 h at 37°C in 20 mM potassium phosphate, pH 7.2, 50 mM EDTA, 0.05% sodium azide, 2% n-octyl-glucoside, and 0.5 U endoglycosidase-F (endo-F) in a volume of 50 µl. To determine the number of N-linked carbohydrate attachment sites on N-CAM, samples were treated with endo-F as above except that 1:4 serial dilutions of enzyme were used to sequentially digest the sugar chains from the protein (Elder and Alexander, 1982; Crossin et al., 1984). For storage, samples were frozen in a dry ice-ethanol bath and kept at -80°C.

Immunoblots - Approximately 200 µg of total protein or 30 µg affinity-purified N-CAM was fractionated by SDS-PAGE (Laemmli, 1970) on 4% gels and transferred to nitrocellulose (Towbin et al., 1979). Non-specific binding was blocked by incubation in TBS-5% BSA for 2 h. Primary antibody, wheat germ agglutinin-HRP (WGA-HRP) conjugate, or biotinylated peanut (PNA) or jackfruit (jacalin) lectin (Vector Laboratories) diluted to 10 µg/ml in TBS-1% BSA was incubated with the blot overnight. Blots were washed 2x10 min in

TTBS and 3x10 min in TBS. For biotinylated-lectin blots, streptavadin-alkaline phosphatase conjugate (1:5000 in TBS-1% BSA) was added for 1 h, the blot was washed as above, and developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitro blue tetrazolium (NBT). For WGA-HRP blots, the blot was developed directly using 4-chloro-1-napthol and hydrogen peroxide. For all lectin blots, samples were pre-digested with neuraminidase to expose terminal sugar residues and enhance lectin binding (Russell et al., 1984). All other blots were incubated with an alkaline phosphatase- or HRP-conjugated secondary antibody (diluted 1:15000 or 1:3000 in TBS-1% BSA, respectively), washed as above, and visualized using BCIP and NBT (for alkaline phosphatase) or 4-chloro-1-napthol and hydrogen peroxide (for HRP) as substrates.

Northern Blot Analysis - Total cytoplasmic RNA was prepared using the cesium trifluoroacetate method (Okayama et al., 1987). 5 µg were fractionated by electrophoresis on 0.5% agarose gels in the presence of formaldehyde and transferred to nitrocellulose (Maniatis et al., 1982). Blots were hybridized overnight at 55°C with a ^{32}P-dCTP-labelled (New England Nuclear, 3000Ci/mmol) DNA probe prepared with a random primers labelling kit (BRL) according to manufacturer's instructions, using a 1210-bp PstI fragment from pEC208 (Hemperly et al., 1986a) as a template. Blots were washed to 0.1 x SSC at 65°C and exposed to RX X-ray film (Fuji) at -80°C.

cDNA Synthesis and PCR - Oligonucleotides with the following sequences were synthesized by Dr. K. Roy (Department of Microbiology, University of Alberta): Oligo 1 (TGTTTTTTCTCGG-AGCCGCA); Oligo 6 (AGCAGAGTACATCTGCATCG); Oligo 8 (TTGAGA-

GTCAGGGACGATAC); Oligo 11 (GGTGCCCATCCTCAAATACA); Oligo 13 (ATGGAGTTTCCGTCTTCTCC); Oligo 19 (CAGATTTGTCTTCTACTGGG); Oligo 3' (TCCCATTCTCACTGGTGTAA). Oligos 1, 6, 11, and 3' are identical to the sense strand of N-CAM cDNA. Oligos 1, 6, and 11 hybridize to the antisense strand approximately 30-bp 5' of the splice junctions between exons 1-2, exons 6-7, and exons 11-12, respectively. Oligo 3' hybridizes to the antisense strand 118-bp upstream of the poly-A tail. Oligos 8, 13, and 19 are antisense, and hybridize to N-CAM mRNA about 30-bp 3' of the splice junctions between exons 7-8, exons 12-13, and exons 17-19 (in 140 kD N-CAM) or 18-19 (in 180 kD N-CAM) (Owens et al., 1987) (see Fig. II-6A). All of the N-CAM sequences were obtained from GenBank, release 61. Two additional primers, pBA9 (GACTCGAGTCGACATC-GAT_{17}) and pBA10 (GACTCGAGTCGACATCG) (gifts from Mr. Phillip Barker, Department of Cell Biology and Anatomy, University of Alberta) were used for specific first strand cDNA synthesis (pBA9) (see below) and for RACE PCR (pBA10) (Frohman et al., 1988) in conjunction with Oligo 3'.

Total cellular RNA (isolated as above) was used as a template for first strand cDNA synthesis. Briefly, 15 µg of total RNA was coprecipitated with 300 ng of random primers, oligo-dT, or primer pBA9, dried, and dissolved in 10 µl annealing buffer (250 mM KCl, 10 mM Tris, pH 8.3, at 50°C). The mixture was denatured for 10 min at 65°C and allowed to cool to room temperature. Then 16.5 µl of reverse transcriptase mix (24 mM Tris, pH 8.3 at 50°C, 16 mM $MgCl_2$, 8 mM DTT, 2 mM each dNTP, 10 U RNasin, 20 U AMV reverse transcriptase) was added to each annealing reaction, and incubated at

50°C for 1 h. 2 µl of this reaction mix was used directly as a template for amplification by PCR under the following conditions in a 100 µl volume: 50 mM KCl, 10 mM Tris, pH 9.0, 0.01% gelatin, 200 µM each dNTP, 1.5 mM $MgCl_2$, 40 pmol each primer, and 2.5 U Taq polymerase (Promega). Samples were taken through 30 PCR cycles as described previously (Small and Akeson, 1990). 20 µl aliquots were loaded on agarose gels and visualized by post-staining for 30 min with 0.75 µg/ml ethidium bromide. Gels were Southern blotted onto Genescreen Plus (Dupont), and probed with a ^{32}P-dCTP-labelled DNA probe prepared using the 3556-bp EcoR1 insert of pEC208 (Hemperly et al., 1986a) as a template, as described above.

Results

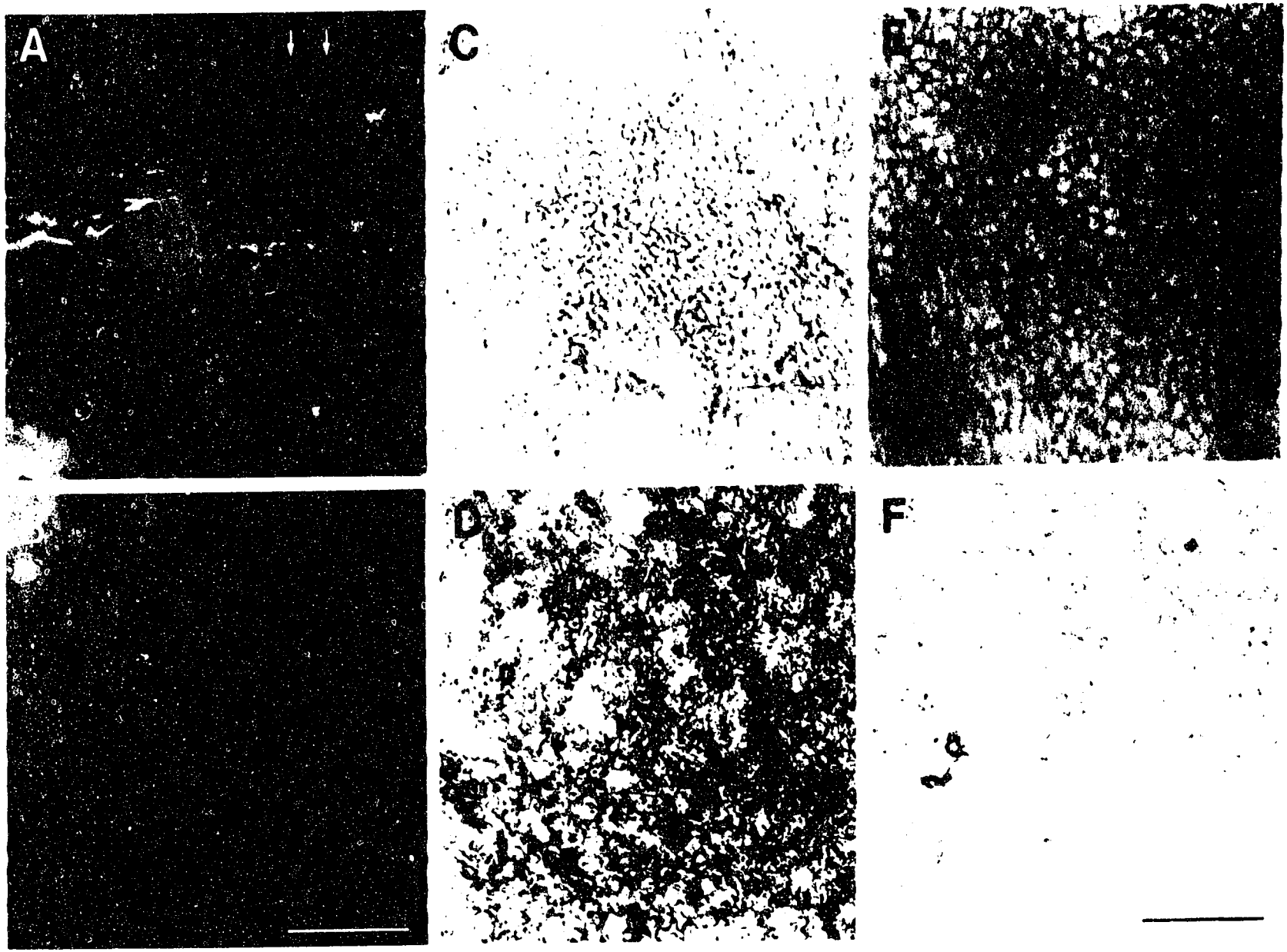
The terminology we use in describing feather structures and the processes involved in feather formation is as outlined by Chuong and Edelman (1985a) and Lucas and Stettenheim (1972).

Distribution of N-CAM and PSA in Developing Feathers

We examined the expression of N-CAM and PSA in developing feather rudiments from stage 28 (E6) to stage 44 (E18) to determine the expression of these molecules prior to (stage 28-29) and during (stage 30-44) feather development.

Prior to the formation of discernible feather structures in the dorsal skin, we found that N-CAM was expressed at low levels on cells of the epidermis and dermis at stage 29 (E6). As shown in Fig. II-1A, both epidermal and dermal cells were N-CAM-positive. Wholemounts of separated epidermis (Fig. II-1C) and dermis

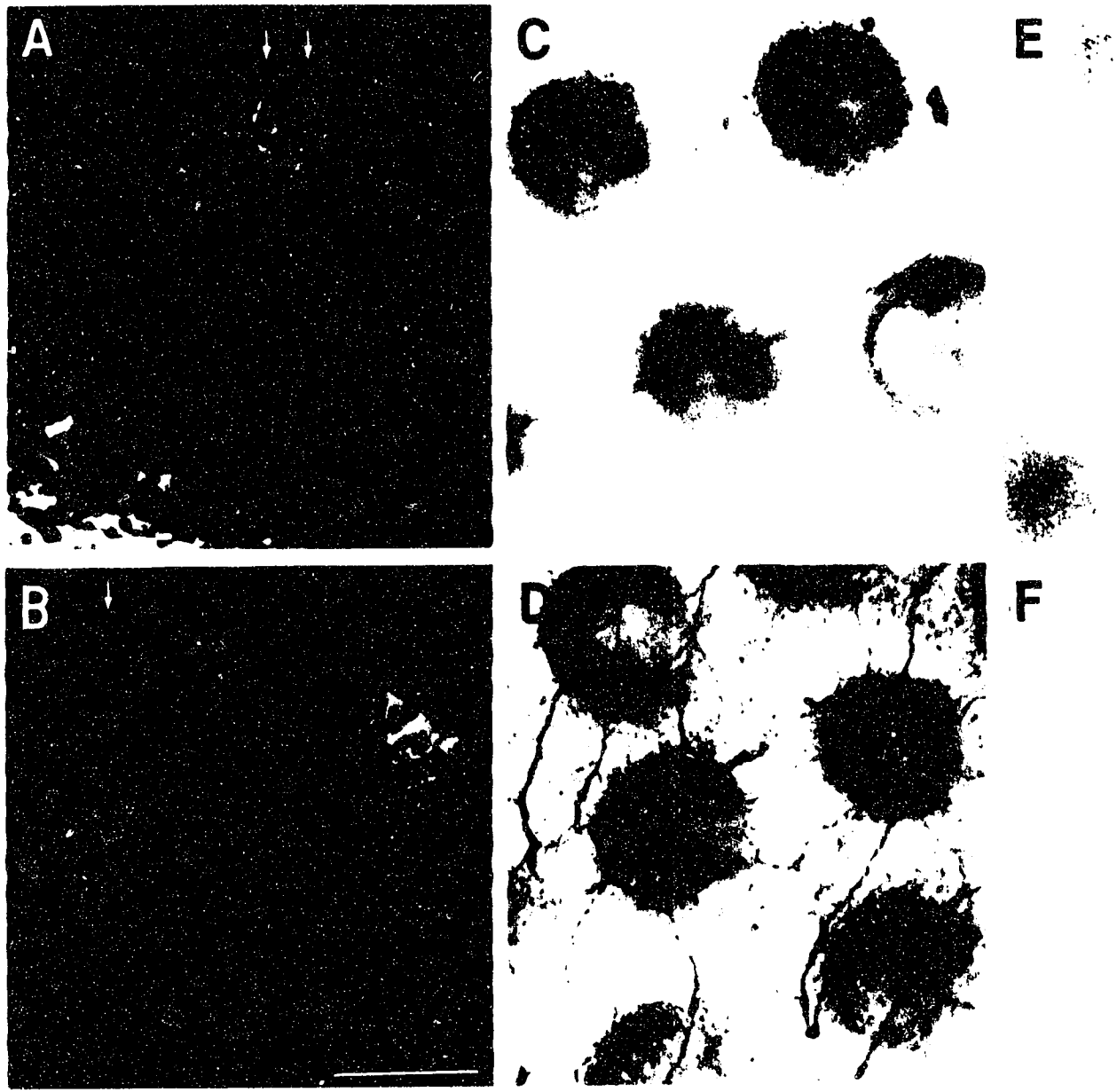
Figure II-1. Immunofluorescent staining of embryonic skin sections (A-B) and immunoperoxidase staining of skin wholemounts (C-F) at stage 29 (E6) with anti-N-CAM CAM6 mAb (A, C, D), anti-PSA 5A5 mAb (B), anti-L-CAM 7D6 mAb (E), or an irrelevant mAb (F). (A) N-CAM is expressed throughout both epidermis and dermis as determined by staining with anti-N-CAM mAb. Arrows denote upper boundary of the epidermis. (B) PSA is not expressed in either epidermis or dermis at this stage. (C) Separated epidermis stained with anti-N-CAM mAb, showing uniform distribution of N-CAM. (D) Separated dermis stained with anti-N-CAM mAb, showing scattered distribution of dermal N-CAM. (E) Separated epidermis stained with anti-L-CAM mAb to verify N-CAM epidermal staining has the same pattern as L-CAM staining. (F) Whole-skin stained with irrelevant mAb against bile cannaliculi. Scale bars = 50 μm.


A
C
E
D
F

(Fig. II-1D) likewise showed that N-CAM was present throughout both tissue layers. Examination of epidermal N-CAM staining at high magnification showed that N-CAM was expressed over the entire surface of the epidermal cells (data not shown). The specificity of N-CAM staining was demonstrated by staining with an irrelevant mAb (Fig. II-1F). As well, several different anti-N-CAM antibodies (monoclonal and polyclonal) gave the same staining pattern (data not shown). L-CAM is present only on epidermal cells in developing feathers (Chuong and Edelman, 1985a), so we verified that N-CAM staining in the epidermis was in the same pattern as L-CAM staining (Fig. II-1E). L-CAM staining in the epidermis showed the same pattern of expression for all ages of skin examined (data not shown). PSA was not detectable in either epidermis or dermis at this stage (Fig. II-1B).

During the formation of feather rudiments (stage 30-35, E7-E9), N-CAM expression changed from being distributed throughout the epidermis and dermis (as in Fig. II-1) to a focused distribution on cells of the developing epidermal placodes and dermal condensations (Fig. II-2A, C, and E). Specifically, N-CAM that was initially present on a loose collection of dermal cells became localized predominantly to dermal cells forming the condensations (Fig. II-2, A and C). Our findings, in conjunction with previous observations (Chuong and Edelman, 1985a), showed that N-CAM expression in the epidermis changed in a similar fashion; we found that from stage 30-34 (E7-E8) N-CAM was present throughout the epidermis (identical to Fig. II-1, A and C) but became localized to cells of the epidermal placodes by

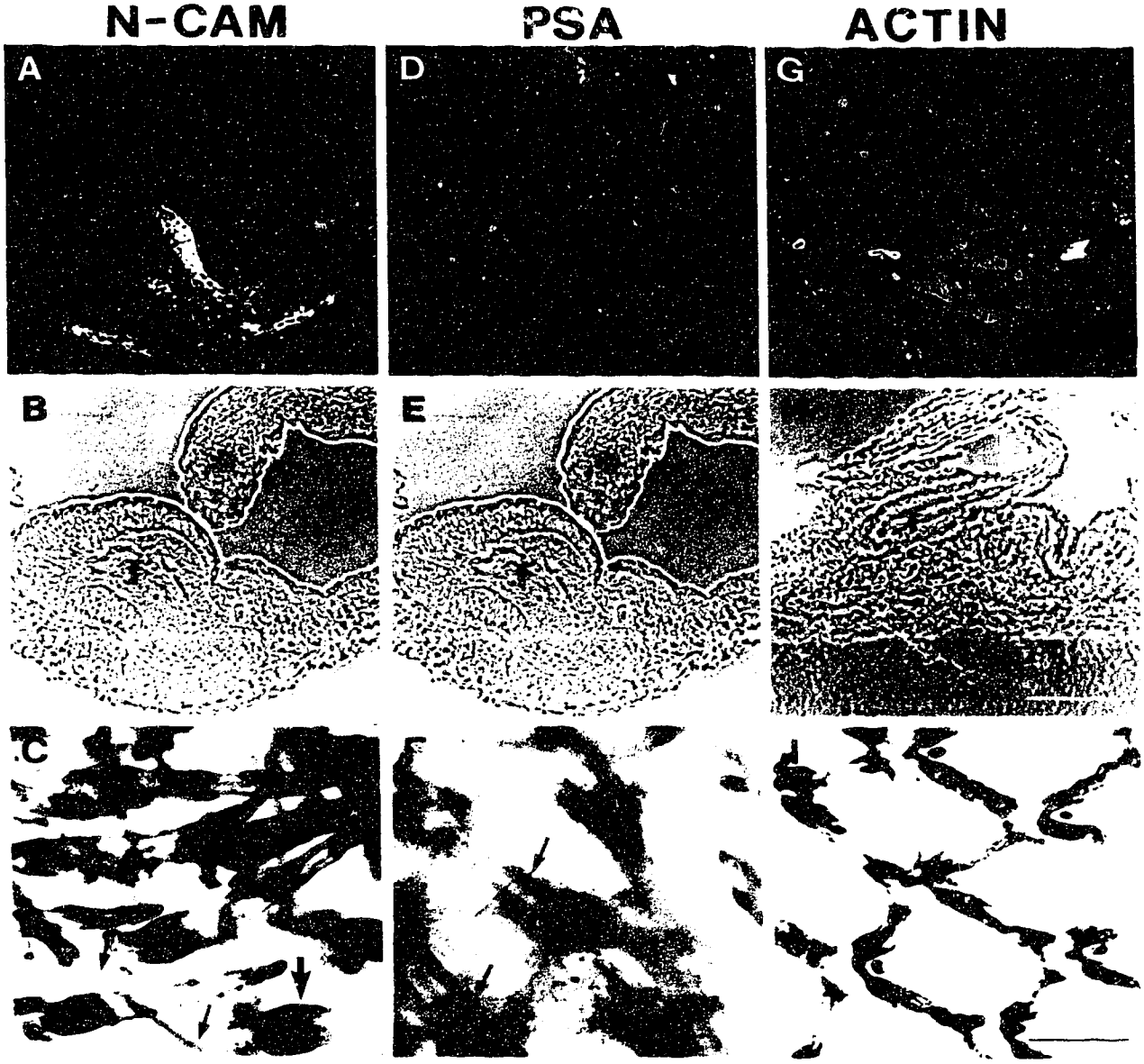
Figure II-2. Immunofluorescent staining of skin sections (A-B) and immunoperoxidase staining of skin dermal (C-D) or epidermal (E-F) wholemounts at stage 35 (E9) with anti-N-CAM CAM6 mAb (A, C, E) or anti-PSA 5A5 mAb (B, D, F). At this stage the expression of N-CAM is now localized to the dermal condensations (A & C) and epidermal placodes (A & E) of the feather rudiments. Note that Fig. II-2A represents N-CAM staining in a single condensation; localization of N-CAM to the dermal condensations is shown by the absence of staining in the surrounding dermis in Fig. II-2C. Arrows indicate upper margin of epidermis. PSA is first expressed in the dermis (B & D) at this stage, but is not present in the epidermis at this (E & F), or any other stage we examined. Skins are oriented with cephalic to the left and caudal to the right. Scale bars = 50 μm (A-B) and 200 μm (C-F).


A
B
C
D
E
F

stage 35 (E9) (Fig. II-2, A and E). It was at stage 35 (E9), when N-CAM was strongly expressed by cells of the epidermal placode and dermal condensation, that we first detected PSA in the dermal condensations (Fig. II-2, B and D). Both N-CAM and PSA were localized predominantly to the cephalic side of the condensation (Fig. II-2, C and D). PSA was not present in the epidermis at this stage (Fig. II-2F), nor was it detected in this tissue layer at any stage of feather development examined.

During the remainder of feather development (stage 36-44, E10-E18), the epidermis invaginates to form a double-layered follicle and the feather rudiments elongate into filaments (Lucas and Stettenheim, 1972). We found that N-CAM was expressed in the elongating feather filaments as previously described (Chuong and Edelman, 1985a) and was also present on cells at the base of the filaments (Fig. II-3, A and C). Staining with an anti-α-actin antibody specific for smooth muscle (Fig. II-3, G and I) and with an anti-tropomyosin antibody that recognized skeletal muscle (data not shown) demonstrated that these cells were smooth muscle. As filament formation progressed, N-CAM was expressed more strongly on the smooth muscle cells and the staining appeared identical to that in Fig. II-3I (data not shown). PSA was expressed in the dermal condensations until stage 36 (E10) (as in Fig. II-2, B and D) and was then localized to the smooth muscle cells for all subsequent stages of feather formation examined (stage 37-44, E11-E18) (Fig. II-3, D and F). As with N-CAM, the expression pattern of PSA shifted from being

Figure II-3. Immunofluorescent staining of skin sections (A, D, G), phase contrast photographs of the same fields (B, E, H), and immunoperoxidase staining of whole-skins (C, F, I) at stage 38 (E12). Staining is with anti-N-CAM CAM6 (A-C), anti-PSA 5A5 (D-F), or anti-α-actin αsm-1 mAb (G-I). N-CAM is expressed in both the filaments (f) (* is an oblique section of a filament) and in the underlying dermis both within the filament (large arrows) and on developing smooth muscle (small arrows) (A & C). PSA is expressed only on cells in the dermis that are forming smooth muscle (small arrows) (D & F). Staining with anti-α-actin mAb (G & I) shows the dermal smooth muscle cells that will become the erector muscles of the feathers. Scale bars = 200 μm (C, F and I) and 50 μm for all other photographs.

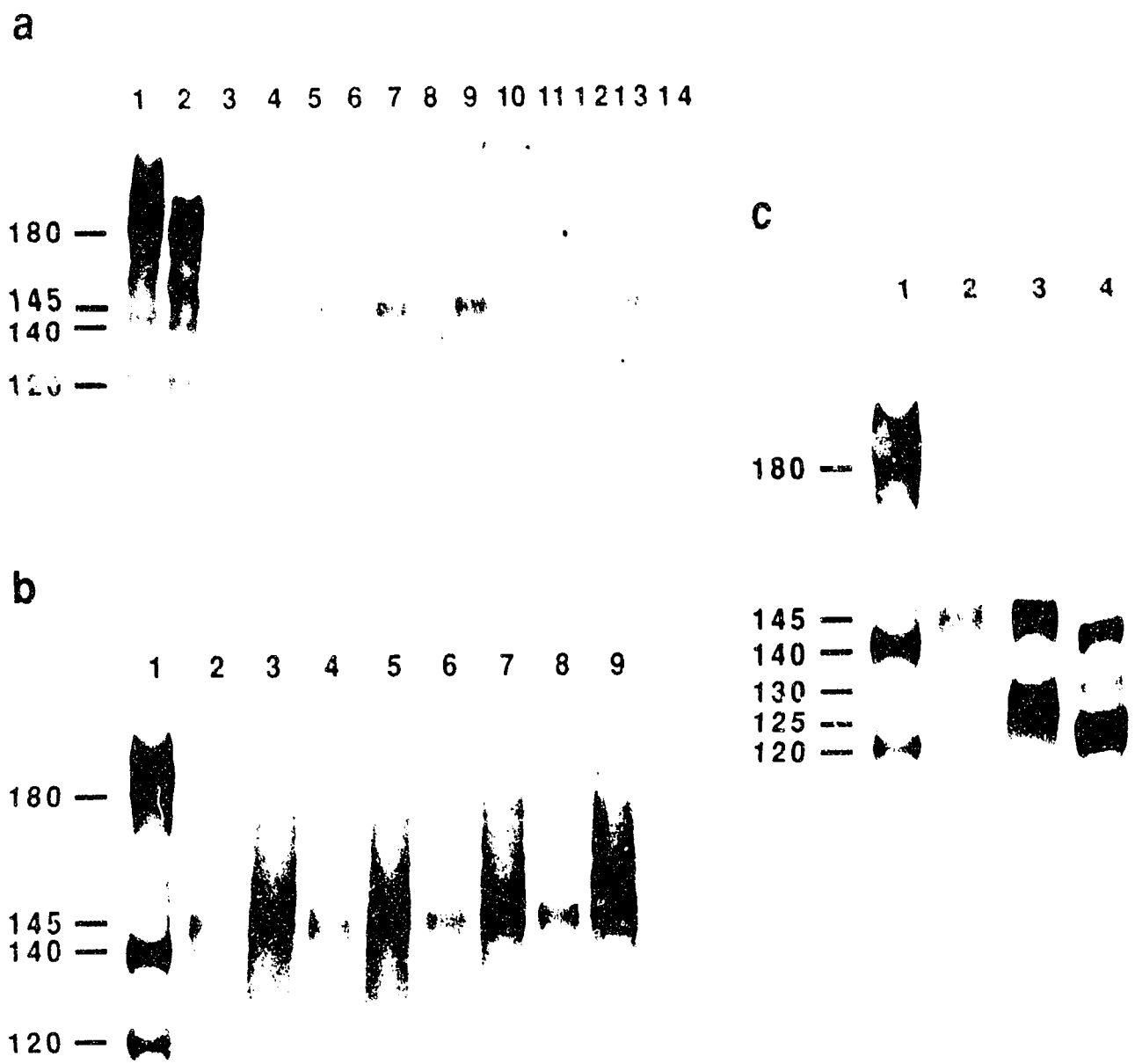

N-CAM
PSA
ACTIN
A
D
G
B
E
C

at the base of the filaments (Fig. II-3F) to being on smooth muscle cells in the dermis (as in Fig. II-3I) (data not shown). PSA was not detected in the feather filaments at any stage.

Immunoblot analysis of N-CAM and PSA in developing feathers

To determine which molecular forms of N-CAM were present in developing feathers, we used Western blot analysis to compare immunoaffinity purified skin N-CAM, and N-CAM from crude homogenates of skin and filaments alone, to N-CAM isolated from embryonic brain (Fig. II-4a). Brain N-CAM from stage 41 (E15) embryos showed a broad band of high molecular weight (180-250 kD), indicating the presence of sialylated N-CAM (Fig. II-4a, lane 1). Removal of the PSA revealed three bands of 180, 140, and 120 kD (Fig. II-4a, lane 2). Immunoaffinity purified N-CAM from stage 41 (E15) skin showed a broad band extending upwards from 145 kD (Fig. II-4a, lane 3), which resolved into a single band of M_r=145 kD upon removal of PSA (Fig. II-4a, lane 4, compared to lane 2). The same 145 kD polypeptide was detected throughout feather development (Fig. II-4a, lanes 5 to 14). Since filaments do not contain any muscle tissue (see above), the 145 kD band seen in developing filaments could not be solely due to contamination of the homogenates with muscle that expresses a previously reported variant of N-CAM (Prediger et al., 1988). PSA was not detected in the feather filaments (Fig II-4a, lanes 11 and 12). No N-CAM could be detected in stage 44 (E18) feather filaments, which are essentially acellular (Lucas and Stettenheim, 1972) (data not shown). The small amounts

Figure II-4. Western blot of N-CAM from (a) skin at different stages of development, (b) separated epidermis and dermis and, (c) brain, skin, heart and skeletal muscle. (a) Affinity-purified N-CAM from stage 41 (E15) skin appears as a broad band extending upwards from 145 kD (lane 3) which, upon removal of PSA, resolves into single band of M_r=145 kD (lane 4) compared to the 180, 140, and 120 kD polypeptides in brain (lane 1, without boiling; lane 2, with boiling). The same M_r=145 kD polypeptide is seen throughout feather development (all paired lanes represent N-CAM without and with boiling, respectively) (E7 skin, lanes 5-6; E10 skin, lanes 7-8; E14 skin, lanes 9-10; E14 filaments alone, lanes 11-12; E18 skin, lanes 13-14). Comparison of N-CAM in E14 skin, without (lane 9) and with boiling (lane 10), to N-CAM in E14 filaments, without (lane 11) and with boiling (lane 12), shows that filament N-CAM does not contain PSA. (b) The epidermis also contains the M_r=145 kD polypeptide, and epidermal N-CAM does not contain PSA, unlike N-CAM from the corresponding dermis (lane 1 is E15 brain N-CAM; lanes 2, 4, 6, and 8 are E6-E9 epidermal N-CAM, respectively; lanes 3, 5, 7, and 9 are E6-E9 dermal N-CAM). (c) A similar-sized M_r=145 kD N-CAM polypeptide is seen in skin, heart, and skeletal muscle (lanes 2, 3, and 4) compared to the 140 kD polypeptide in brain (lane 1) (all samples were boiled). Anti-N-CAM 802 polyclonal antibody was the primary antibody for all blots shown. Marks on the left-hand side of the blots indicate the size of the protein bands in kD.


a
1 2 3 4 5 6 7 8 9 10 11 12 13 14
180
145
140
120
b
1 2 3 4 5 6 7 8 9
180
145
140
120
c
1 2 3 4
180
145
140
130
125
120

of 180 kD N-CAM polypeptide seen in some of the skin extracts is due to nerves present within the dermis; the 180 kD form of N-CAM is known to be restricted to nerves in developing feathers (Prieto et al., 1989).

To verify that the difference between the 140 kD N-CAM polypeptide in brain and the 145 kD N-CAM polypeptide in developing feathers was not due to a gel artifact, we ran multiple parallel lanes of immunoaffinity purified N-CAM from brain and skin on a gel and immunoblotted with a polyclonal anti-N-CAM antibody. We consistently found a N-CAM polypeptide in skin that was 145 kD compared to the 140 kD polypeptide in embryonic brain (data not shown).

In order to demonstrate the presence of the 145 kD N-CAM polypeptide and the absence of PSA in the epidermis, we performed Western blot analysis on extracts made from stage 29-35 (E6-E9) epidermis and compared them to those from the corresponding dermis. As Fig. II-4b shows, a single 145 kD N-CAM polypeptide was present in the epidermis from stage 29 (E6) to stage 35 (E9) (lanes 2, 4, 6, and 8), and did not contain any PSA, unlike N-CAM in the corresponding dermis (lanes 3, 5, 7, and 9).

The presence of a M_r=145 kD N-CAM polypeptide in developing feathers raised the question of whether this polypeptide was the same size as a M_r=145-150 kD polypeptide reported in other non-neuronal tissues such as embryonic heart and skeletal muscle (Murray et al., 1986; Moore et al., 1987). Comparison of the N-CAM polypeptides present in embryonic chick brain, feather filaments, heart, and skeletal muscle (Fig. II-4c) showed that all three non-neuronal tissues expressed a similar-sized M_r=145 kD polypeptide

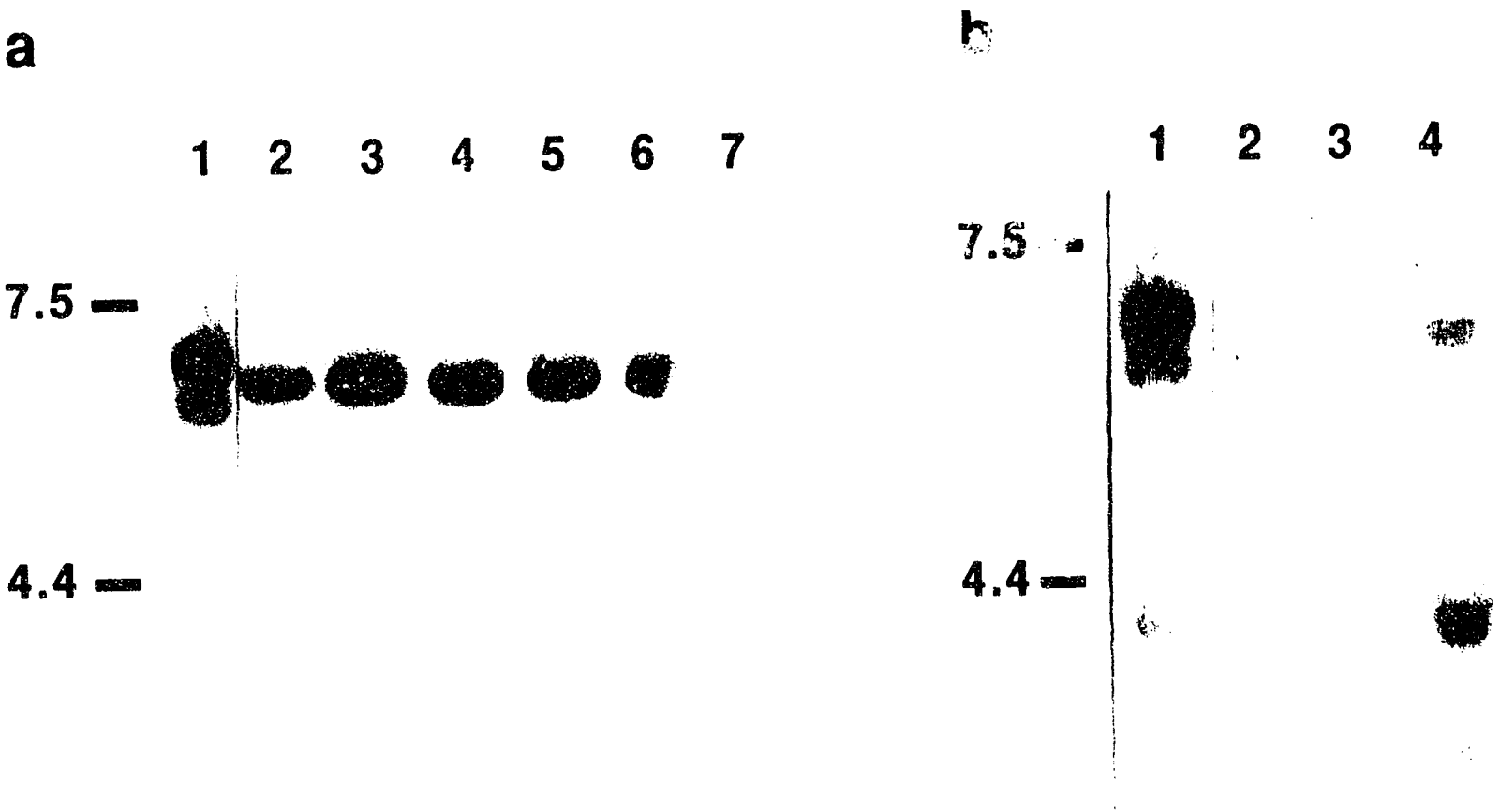
(Fig. II-4c, lanes 2-4) compared to the 140 kD polypeptide in brain (lane 1). Heart and skeletal muscle also expressed several smaller polypeptides of M_r=130 and 120-125 kD, as previously reported (Murray et al., 1986; Moore et al., 1987) (Fig. II-4c, lanes 3 and 4).

Northern blot analysis of N-CAM mRNA in developing feathers

We compared the N-CAM mRNA species present in feathers with those present in embryonic brain to determine if the M_r=145 kD polypeptide in feathers was encoded by a larger mRNA than the 140 kD polypeptide in brain. Throughout feather development, we detected a single mRNA species of 6.4 kb (Fig. II-5a, lanes 2 to 6), compared to 6.9, 6.1, and 4.0 kb mRNAs in embryonic brain (Fig. II-5a, lane 1). Embryonic liver contains little N-CAM after stage 30 (E7) and served as a negative control (Fig. II-5a, lane 7).

The presence of a 6.4 kb N-CAM mRNA species in developing feathers raised the question of whether a similar-sized transcript was present in heart and skeletal muscle, which also expressed a M_r=145 kD N-CAM polypeptide. Comparison of N-CAM mRNAs present in stage 41 (E15) feather filament, heart, and skeletal muscle (Fig. II-5b, lanes 2-4) showed that all three non-neuronal tissues contain a 6.4 kb mRNA, compared to the 6.1 kb mRNA in stage 41 (E15) brain (Fig. II-5b, lane 1).

Figure II-5. Northern blots (a) of N-CAM mRNA expressed during feather development (lane 1, E15 brain; lane 2, E7 skin; lane 3, E10 skin; lanes 4-5, E14 skin and filament alone; lane 6, E18 skin; lane 7, E15 liver), and (b) comparing N-CAM mRNA in E15 brain (lane 1), filament (lane 2), heart (lane 3), and skeletal muscle (lane 4). (a) A single 6.4 kb mRNA is present during feather formation and, (b) a similar-sized 6.4 kb mRNA is seen in all three non-neuronal tissues. In each case, 5 ug total RNA was hybridized to a DNA probe prepared from the 1210-bp Pst I fragment of pBS208. Size markers (in kb) are at the sides of the blots.


a
1 2 3 4 5 6 7
7.5
4.4
b
1 2 3 4
7.5
4.4

PCR analysis of exon splicing pattern and polyadenylation sites in feather N-CAM mRNA

In addition to the alternative splicing which generates the major neuronal N-CAM polypeptides (Owens et al., 1987) (see Fig. II-6A), splicing that alters shorter segments of the N-CAM polypeptide chains also occurs between exons 7-8 and 12-13 (Dickson et al., 1987; Gower et al., 1988; Prediger et al., 1988; Small et al., 1988; Santoni et al., 1989; Thompson et al., 1989; Small and Akeson, 1990; Reyes et al., 1991; Zorn and Krieg, 1992). In embryonic chicken, the only known alternative splice is a 93-bp insert, composed of four exons, that is present between exons 12-13 in heart and skeletal muscle N-CAM mRNA. This insert was detected by screening an embryonic heart cDNA library (Prediger et al., 1988).

The larger size of the 6.4 kb N-CAM mRNA species in feathers compared to the 6.1 kb mRNA in brain suggested that alternatively spliced exons might be present within the feather N-CAM mRNA. To determine whether or not the 93-bp alternative splice, or novel splices, were present in N-CAM of developing feathers and accounted for the larger size of the 6.4 kb mRNA, we used the polymerase chain reaction (PCR) to amplify and compare selected regions of N-CAM mRNA in embryonic chicken brain, filaments, heart, and skeletal muscle.

In order to find out where in the 6.4 kb N-CAM mRNA any alternative splices were present, we compared the PCR products generated by amplifying the regions between exons 1-8, 6-8, 6-13, 11-13, and 11-19 (see Fig. II-6A). The sizes of the products generated from brain RNA using our primers are 1086-bp for exons 1-8,

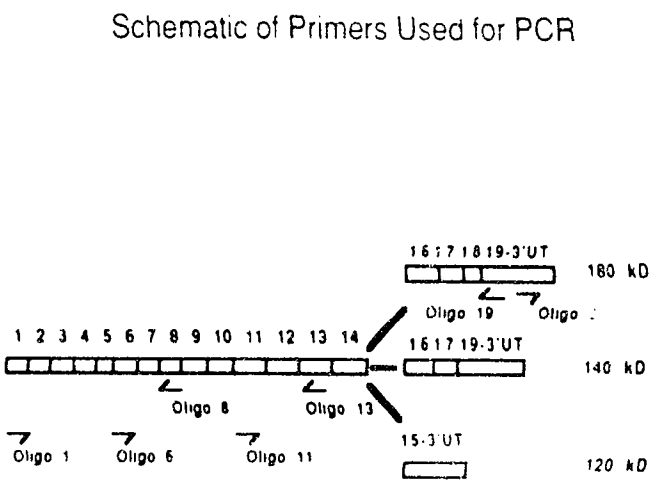
272-bp for exons 6-8, 1004-bp for exons 6-13, 270-bp for exons 11-13, and 871-bp (for 140kD N-CAM) or 1654 bp (for 180kD N-CAM) (Owens et al., 1987) for exons 11-19.

Comparison of the products from exons 1-8 (Fig. II-6C, lanes 1-4) and exons 6-8 (Fig. II-6D, lanes 1-4) showed that all four tissues lack alternative splices between exons 1-8; single bands of 1086-bp and 272-bp were detected in each tissue for the regions between exons 1-8 and 6-8, respectively. Restriction digests of the exons 1-8 and 6-8 PCR products confirmed that there were no differences in the products obtained from the four tissues (data not shown). Thus, unlike the alternative splicing of the 30-bp VASE (or π) insert at the exon 7-8 junction in rat brain and heart (Small et al., 1988; Small and Akeson, 1990; Reyes et al., 1991) and in many frog tissues (Zorn and Krieg, 1992), we find no evidence for alternative splicing in this region of chicken N-CAM, in agreement with previous investigations (Small et al., 1988). If VASE or other inserts are present in this region of chicken N-CAM, they must either be present in the N-CAM mRNA at levels below the sensitivity of our methods or be expressed at specific developmental stages that were not examined.

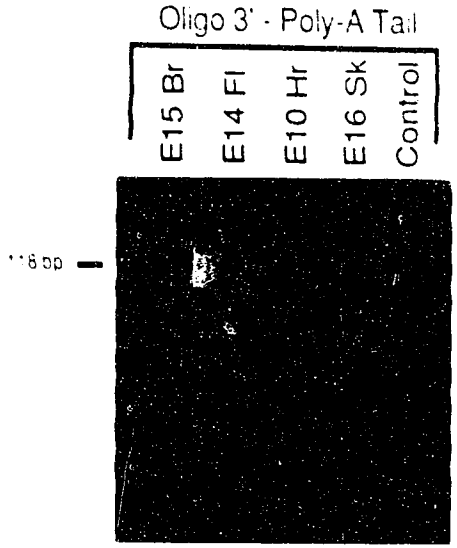
Amplification of the region between exons 6-13, 11-13, and 11-19 revealed an alternative splice in the non-neuronal tissues and the absence of this splice in brain. In particular, in all three non-neuronal tissues: (a) two bands of 1004-bp and 1097-bp were generated by amplifying the region between exons 6-13 (Fig. II-6C, lanes 6-8); (b) two bands of 270-bp and 362-bp were generated for the region between exons 11-13 (Fig. II-6D, lanes 6-8) and; (c) two bands of 871-bp and 964-bp were generated for the region between

Figure II-6. Analysis of exon splicing pattern and polyadenylation sites in skin N-CAM mRNA by using the polymerase chain reaction (PCR). (A) Schematic illustration of primers used for PCR reactions, and their relation to the mRNAs encoding the 180, 140, and 120 kD brain N-CAM polypeptides. Numbers above open boxes represent exons 1-19 as indicated. Exons 1-14 are shared by all N-CAM mRNAs. Exon 15 is present only in the 120 kD form, and exon 18 is present only in 180 kD form, as shown at the right. 3'-UT represents the 3' untranslated region up to and including the poly-A tail. (B) RACE PCR amplification from oligo 3' to the poly-A tail of E15 brain (Br), E14 filament (Fl), E10 heart (Hr), and E16 skeletal muscle (Sk) mRNAs. Controls with no template added did not generate any bands. (C) PCR amplification of the regions between exons 1-8, exons 6-13, and exons 11-19 in brain (Br), filament (Fl), heart (Hr), and skeletal muscle (Sk) mRNAs. (D) PCR amplification of the regions between exons 6-8 and exons 11-13 in brain (Br), filament (Fl), heart (Hr), and skeletal muscle (Sk) mRNAs. Controls with no template added did not generate any bands. For each gel, the sizes of the PCR products (in bp) are indicated at the left.

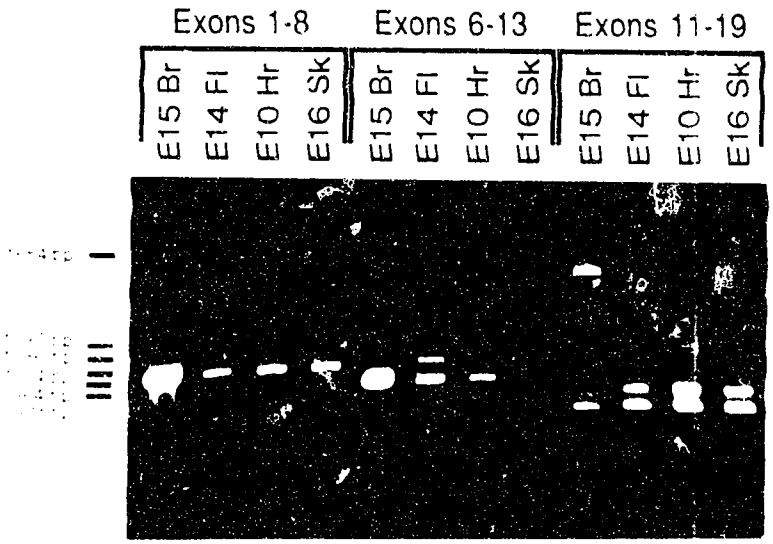
A


Schematic of Primers Used for PCR
16 17 18 19-3'UT
180 kD
Oligo 19
Oligo
1 2 3 4 5 6 7 8 9 10 11 12 13 14
16 17 19-3'UT
140 kD
Oligo 8
Oligo 13
Oligo 1
Oligo 6
Oligo 11
15-3'UT
120 kD

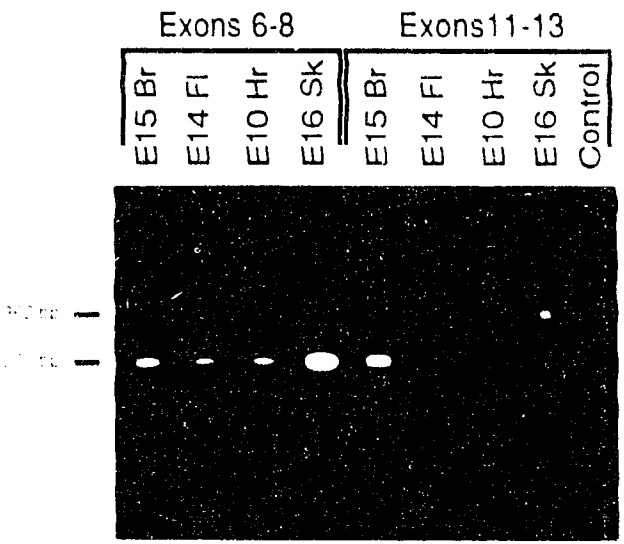
B


Oligo 3' - Poly-A Tail
E15 Br
E14 Fl
E10 Hr
E16 Sk
Control

C


Exons 1-8
Exons 6-13
Exons 11-19
E15 Br
E14 Fl
E10 Hr
E16 Sk

D


Exons 6-8
Exons11-13
E15 Br
E14 Fl
E10 Hr
E16 Sk
Control

exons 11-19 (Fig. II-6C, lanes 10-12). The two bands of 871-bp and 1654-bp seen in Fig. II-6C, lane 9 (brain, exons 11-19) represent the mRNAs with and without exon 18, the splice that generates the 180 kD N-CAM polypeptide in brain (Owens et al., 1987) (see Fig. II-6A). Fig. II-6D, lane 9, represents a typical control in which no template was added to the PCR reaction. Restriction digests of the 6-13, 11-13, and 11-19 PCR products showed that a 93-bp insert was present between exons 12-13 (data not shown). We did not find evidence for differential expression of the four exons comprising the 93-bp insert, unlike the four exons comprising the 108-bp MSD1 insert in rat heart (Reyes et al., 1991) and the two exons comprising the 18-bp MSD insert in frog (Zorn and Krieg, 1992).

The 93-bp insert could not account for the observed difference in size of the 6 mRNAs in the non-neuronal tissues compared to the 6.1 kb mRNA in brain because two transcripts, with and without the insert, were present in the non-neuronal tissues based on PCR, yet only a single 6.4 kb band was seen on Northerns. We therefore looked for alternative polyadenylation sites in the 6.4 kb mRNAs that would increase the length of their 3' untranslated region. Rapid amplification of cDNA ends (RACE) PCR (Frohman et al., 1988) was used to selectively amplify the region between Oligo 3' (see Fig. II-6A) and the poly-A tail of the mRNAs (Fig. II-6B). A single 118-bp product was generated in all four tissues by amplifying this region (Fig. II-6B, lanes 1-4), indicating the same polyadenylation site was used in the 6.4 kb non-neuronal and 6.1 and 6.9 kb neuronal mRNAs. Fig. II-6B, lane 5, is a control with no template added to the PCR

reaction. Differential polyadenylation therefore does not account for the observed larger size of the [illegible] kb non-neuronal mRNAs compared to the 6.1 kb mRNA in brain.

Analysis of oligosaccharides attached to skin N-CAM

The identification of two 6.4 kb skin N-CAM mRNAs, with and without the 93-bp insert, means that two polypeptides, differing in size by about 3.4 kD, are probably present on immunoblots of skin N-CAM. As Fig. II-4a shows, these two polypeptides are not resolved by our gel system. Thus, the presence versus absence of the 93-bp insert can not account for the observed 5 kD size difference between the skin and brain polypeptides.

An alternative explanation for the increased size of the skin polypeptides is the attachment of carbohydrate to the proteins. N-CAM in chick brain has several sites for N-linked carbohydrate attachment, three of which are known to be glycosylated (Crossin et al., 1984; Cunningham et al., 1987). O-linked carbohydrate attachment to N-CAM has only been shown to occur on the 108-bp alternative splice MSD1 in human skeletal muscle N-CAM (Walsh et al., 1989). The 93-bp insert present in chicken skin is similar in structure to MSD1 (Prediger et al., 1988) and thus may serve as a site for O-linked attachment. However, since the 93-bp insert does not account for the size difference between the skin and brain polypeptides, O-linked carbohydrate that might be associated with this insert can also not be the basis of the size difference. We therefore first determined whether N-linked carbohydrate was present on the

skin N-CAM proteins by using lectin blot analysis with wheat germ agglutinin (WGA), which recognizes sugar linkages only in N-linked carbohydrate chains (Allen et al., 1973; Naquta and Berger, 1974).

WGA lectin blot analysis showed that N-linked carbohydrate was indeed present on the skin N-CAM polypeptides (Fig. II-7A). WGA bound to many N-linked proteins in whole filament extract, as expected (lane 1). However, in affinity-purified filament N-CAM, WGA bound to a single band corresponding to the 145 kD skin proteins (Fig. II-7A, lane 2; see also Fig. II-7D, lanes 1 and 2). Affinity-purified N-CAM from brain and skeletal muscle were blotted with WGA for controls because the 180, 140, and 120 kD brain N-CAM proteins, and the 145 and 130 kD muscle N-CAM proteins (see Fig. II-7D, lanes 3 and 4) are known to be N-glycosylated (Cunningham et al., 1983; Crossin et al., 1984; Barton et al., 1988). All three brain proteins were recognized by WGA as expected (Fig. II-7A, lane 3). Interestingly, in the skeletal muscle N-CAM, WGA bound to the 145 and 130 kD N-CAM proteins, but also bound to two other proteins of 155 and 190 kD (lane 4). These latter two proteins are not recognized by antibodies to N-CAM on Western blots (Fig. II-7D, lane 4). This suggests that other N-glycosylated proteins may interact with muscle N-CAM and remain attached to it during affinity purification. In support of this idea, it was recently found that N-CAM co-localizes with another cell adhesion molecule, N-cadherin, in avian skeletal myoblasts (Soler and Knudsen, 1991). Although neither the 155 kD nor the 190 kD band detected by WGA matches the known size of N-cadherin, it is possible that these two

rudiments and lead to abnormal filaments. Surprisingly, neither the antibody nor the endo-N treatment led to any apparent changes in the morphology or histology of the feather rudiments or filaments, suggesting that the normal cellular processes which occur during feather morphogenesis were not altered.

As discussed in Chapter III of this thesis, there were a number of possible reasons why the antibody and endo-N treatments did not alter feather development, including technical problems, the existence of redundant adhesive pathways in skin, and the fact that the time of expression of N-CAM does not necessarily coincide with the time that N-CAM is involved in feather development. Technical problems were largely ruled out by verifying that (a) normal feather structures developed using our culture and grafting systems, (b) N-CAM and PSA were expressed the same way in vitro as in vivo, (c) 50 U/ml of endo-N was sufficient to remove all PSA from the cultures and grafts, (d) the absence of PSA in the endo N treated cultures and grafts was due to digestion by endo-N and not to unknown factors, (e) the anti-N-CAM mAb inhibited N-CAM homophilic adhesion, and (f) the anti-N-CAM mAb was able to penetrate and disperse within the skin explants. The existence of redundant adhesion pathways that might have compensated for the loss of N-CAM homophilic binding in skin also seemed unlikely, since antibodies against N-CAM were found to alter feather morphogenesis in a previous study (Jiang and Chuong, 1992) and showed that normal N-CAM-mediated adhesion was crucial for proper feather development. Likewise, the possibility that N-CAM expression and function did not coincide temporally was also

unlikely because the time span examined in the current perturbation study was from stage 30 (E7) to stage 42 (E16), a period covering both rudiment and filament formation. One difference, however, between the current study and the Jiang and Chuong (1992) study was the time of initial application of antibody to the skin explants; in the current study the skins were exposed to antibody at stage 30 (E7) whereas in the other study the skins were exposed to antibody at stage 33 (E8). At stage 30, feather rudiments are just starting to appear in the dorsal skin. In contrast, at stage 33, multiple rows of feather rudiments are already established. The possibility therefore exists that this difference in the time of antibody application might have led to the different results of the two studies. This possibility remains to be tested.

An additional reason why the antibody treatment in the current study may not have caused alterations in feather morphogenesis was the possibility that different regions of the skin N-CAM proteins mediated specific functions such as homophilic binding, interaction with extracellular matrix (ECM), or interaction with other cell surface proteins. Structural studies of N-CAM (Cunningham et al., 1983; Cole et al., 1986; Frelinger and Rutishauser, 1986; Watanabe et al., 1986) have shown that the N-CAM molecule consists of several distinct regions that contain the known cell-cell binding site, cell-ECM binding site, and carbohydrate attachment sites. The possibility therefore existed that these different binding and attachment sites might have had varied importance at different stages of feather development. Additionally, the possibility existed that different regions of the skin N-CAM proteins might have compensated for loss

of function in another part of the proteins. Keeping these ideas in mind, even though the antibody used in the current perturbation experiment was shown to inhibit N-CAM homophilic binding, this function of N-CAM may not have been important for normal feather development, or other regions of the skin N-CAM proteins may have compensated for the loss of binding. If either, or both, of these possibilities were true, then alterations in feather development would not have been caused by our antibody. In contrast, if the antibodies used in the Jiang and Chuong (1992) study bound to different regions of the skin N-CAM proteins that did mediate important functions, or if the function of the bound regions could not be compensated for, then alterations in development would have been caused. In support of this idea, the antibodies used in the Jiang and Chuong study were polyclonal, and therefore likely bound to many different regions on the skin N-CAM proteins. Consequently, it is entirely possible that their antibodies altered different functions of the skin N-CAM proteins than our antibody and therefore had a different effect on feather morphogenesis.

In terms of the endo-N treatment, one explanation as to why removal of PSA did not lead to any changes in feather morphogenesis was the possibility that the PSA present in the dermal condensations was not involved in feather morphogenesis but was instead involved in development of the feather erector muscles within the dermis. Staining of the organ cultures and ChAM grafts with an α-actin mAb showed that smooth muscle cells continued to develop within the dermis in vitro. This indicated that the cells involved in erector muscle formation were present in the skin at the time of removal

from the embryo (stage 30, E7) and subsequently organized themselves into the criss-cross pattern characteristic of mature erector muscle. In the 3-day organ cultures, PSA was expressed in a crescent-shaped pattern in the cephalic region of the feather rudiments. In the 8-day organ cultures, PSA was still expressed in the same crescent-shaped pattern, but bands of cells interconnecting the crescent-shaped areas in different feather rudiments also expressed PSA. This observation suggested that the PSA-staining cells in the feather rudiments of the 3-day cultures might represent smooth muscle precursor cells, and that these cells might not be directly involved in the formation of feathers. If this was indeed the case, then the removal of PSA from the skin explants would not have altered feather development. However, the removal of PSA might have altered the normal development of the smooth muscle cells themselves. Unfortunately, no apparent changes in the development or organization of the smooth muscle cells were observed in either the endo-N or CAM6 mAb treated cultures and grafts.

Clearly, a large number of questions remain about the function of N-CAM in feather morphogenesis. To further our understanding about the role of N-CAM in this developmental process, future experiments should determine whether (a) the two N-CAM proteins in skin have distinct spatiotemporal expression patterns during feather morphogenesis, (b) the 93-bp insert modulates the function of N-CAM in skin, (c) the N-linked carbohydrate present on the skin proteins modulates their function and, (d) antibodies that recognize different regions of the N-CAM proteins have different effects on feather morphogenesis when used both singly and in combination.

Although not directly related to feather development, it would also be interesting to examine in greater detail the function of N-CAM-mediated adhesion in the formation of the feather erector muscles.

References

Acheson, A., Sunshine, J. L. and Rutishauser, U. (1991). N-CAM polysialic acid can regulate both cell-cell and cell-substrate interactions. *J. Cell Biol.* **114**, 143-153.

Allen, A. K., Neuberger, A. and Sharon, N. (1973). The purification, composition, and specificity of wheat germ agglutinin. *Biochem. J.* **131**, 155-162.

Barthels, D., Santoni, M.-J., Wille, W., Ruppert, C., Chaix, J.-C., Hirsch, M.-R., Fontecilla-Camps, J. C. and Goridis, C. (1987). Isolation and nucleotide sequence of mouse N-CAM cDNA that codes for a M_r 79000 polypeptide without a membrane-spanning region. *EMBO J.* **6**, 907-914.

Brackenbury, R. (1992). Personal communication.

Brotman, H. F. (1980). Abnormal morphogenesis of feather structures and pattern in the chick embryo integument. *J. Exp. Zool.* **200**. 107-124.

Chuong, C.-M. and Edelman, G. M. (1985a). Expression of cell-adhesion molecules in embryonic induction. I. Morphogenesis of nestling feathers. *J. Cell Biol.* **101**, 1009-1026.

Cole, G. J., Loewy, A., Cross, N. V., Akeson, R. and Glaser, L. (1986). Topographic localization of the heparin-binding domain of the neural cell adhesion molecule N-CAM. *J. Cell Biol.* **103**, 1739-1744.

Colewell, G., Li, B., Brady-Kalnay, S. and Brackenbury, R. (1991). Control of N-CAM expression in normal and RSV-transformed cells. *J. Cell Biol.* **115**, 69a.

Crossin, K. L., Edelman, G. M. and Cunningham, B. A. (1984). Mapping of three carbohydrate attachment sites in embryonic and adult forms of the neural cell adhesion molecule. *J. Cell Biol.* **99**, 1848-1855.

Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R. and Edelman, G. M. (1987). Neural cell adhesion molecule: Structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* **236**, 799-806.

Cunningham, B. A., Hoffman, S., Rutishauser, U., Hemperly, J. J. and Edelman, G. M. (1983). Molecular topography of the neural cell adhesion molecule N-CAM: Surface orientation and location of sialic acid-rich and binding regions. *Proc. Natl. Acad. Sci. USA* **80**, 3116-3120.

Davidson, D. (1983a). The mechanism of feather pattern development in the chick. I. The time of determination of feather position. *J. Embryol. Exp. Morphol.* **74**, 245-259.

Desbien, X., Queva, C., Jaffredo, T., Stehelin, D. and Vandenbunder, B. (1991). The relationship between cell proliferation and the transcription of the nuclear oncogenes c-myc, c-myb, c-ets-1 during feather morphogenesis in the chick embryo. *Development* **111**, 699-713.

Dickson, G., Gower, H. J., Barton, C. H., Prentice, H. M., Elsom, V. L., Moore, S. E., Cox, R. D., Quinn, C., Putt, W. and Walsh, F. S. (1987). Human muscle neural cell adhesion molecule (N-CAM): Identification of a muscle-specific sequence in the extracellular domain. *Cell* **60**, 1119-1130.

Elder, J. H. and Alexander, S. (1982). Endo-ß-N-acetylglucosaminidase F: Endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. *Proc. Natl. Acad. Sci USA* **79**, 4540-4544.

Frelinger, A. L. and Rutishauser, U. (1986). Topography of N-CAM structural and functional determinants. II. Placement of monoclonal antibody epitopes. *J. Cell Biol.* **103**, 1729-1737.

Frohman, M. A., Bush, M. K. and Martin, J. R. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998-9002.

Goridis, C. and Wille, W. (1988). The three size classes of mouse N-CAM proteins arise from a single gene by a combination of alternative splicing and use of different polyadenylation signals. *Neurochem. Int.* **12**, 269-272.

Hirsch, M.-R., Gaugler, L., Deagostini-Bazin, H., Bally-Cuif, L. and Goridis, C. (1990). Identification of positive and negative regulatory elements governing cell-type-specific expression of the neural cell adhesion molecule gene. *Molec. Cell. Biol.* **10**, 1959-1968.

Hoffman, S. and Edelman, G. M. (1983). Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc. Natl. Acad. Sci. USA* **80**, 5762-5766.

Hoffman, S., Sorkin, B. C., White, P. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B. A., and Edelman, G. M. (1982). Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. *J. Biol. Chem.* **257**, 7720-7729.

Jiang, T.-X. and Chuong, C.-M. (1992). Mechanism of skin morphogenesis. I. Analyses with antibodies to adhesion molecules tenascin, N-CAM, and integrin. *Dev. Biol.* **150**, 82-98.

Lotan, R., Skutelsky, E., Danon, D. and Sharon, N. (1975). The purification, composition, and specificity of the anti-T lectin from peanut (*Arachis hypogaea*). *J. Biol. Chem.* **250**, 8518-8523.

Lyles, J. M., Linnemann, D. and Bock, E. (1984). Biosynthesis of the D2-cell adhesion molecule: Post-translational modifications, intracellular transport, and developmental changes. *J. Cell Biol.* **99**, 2082-2091.

Mahanta, S K., Sastry, M. V. K. and Surolia, A. (1990). Topography of the combining region of a Thomsen-Friedenreich-antigen-specific lectin jacalin (*Artocarpus integrifolia* agglutinin). *Biochem. J.* **265**, 831-840.

Marsh, R. G. and Gallin, W. J. (1992). Structural variants of the neural cell adhesion molecule (N-CAM) in developing feathers. *Dev. Biol.* **150**, 171-184.

Naquta, Y. and Berger, M. M. (1974). Wheat germ agglutinin: Molecular characteristics and specificity for sugar binding. *J. Biol. Chem.* **249**, 3116-3112.

Prediger, E. A., Hoffman, S., Edelman, G. M. and Cunningham, B. A. (1988). Four exons encode a 93-base-pair insert in three neural cell adhesion molecule mRNAs specific for chicken heart and skeletal muscle. *Proc. Natl. Acad. Sci. USA* **85**, 9616-9620.

Reyes, A. A., Small, S. J. and Akeson, R. (1991). At least 27 alternative spliced forms of the neural cell adhesion molecule mRNA are expressed during rat heart development. *Mol. Cell. Biol.* **11**, 1654-1661.

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

Santoni, M. J., Barthels, D., Vopper, G., Boned, A., Goridis, C. and Wille, W. (1989). Differential exon usage involving an unusual splicing mechanism generates at least eight types of N-CAM cDNA in mouse brain. *EMBO J.* **8**, 385-391.

Small, S. J. and Akeson, R. (1990). Expression of the unique N-CAM VASE exon is independently regulated in distinct tissues during development. *J. Cell Biol.* **111**, 2089-2096.

Small, S. J., Haines, S. L. and Akeson, R. A. (1988). Polypeptide variation in an N-CAM extracellular immunoglobulin like fold is developmentally regulated through alternative splicing. *Neuron* **1**, 1007-1017.

Thompson, J., Dickson, G., Moore, S. E., Gower, H. J., Putt, W., Kenimer, J. G., Barton, C. H. and Walsh, F. S. (1989). Alternative splicing of the neural cell adhesion molecule gene generates variant extracellular domain structure in skeletal muscle and brain. *Genes Dev.* **3**, 348-357.

Walsh, F. S., Parekh, R. B., Moore, S. E., Dickson, G., Barton, C. H., Gower, H. J., Dwek, R. A. and Rademacher, T. W. (1989). Tissue specific O-linked glycosylation of the neural cell adhesion molecule (N-CAM). *Development* **105**, 803-811.

Watanabe, M., Frelinger, A. L. and Rutishauser, U. (1986). Topography of N-CAM structural and functional determinants. I. Classification of monoclonal antibody epitopes. *J. Cell Biol.* **103**, 1721-1727.

Wessells, N. K. (1965). Morphology and proliferation during early feather development. *Dev. Biol.* **12**, 131-153.

Zorn, A. M. and Krieg, P. A. (1992). Developmental regulation of alternative splicing in the mRNA encoding *Xenopus laevis* neural cell adhesion molecule (N-CAM). *Dev. Biol.* **149**, 197-205.