ROLE AND REGULATION OF CADHERIN EXPRESSION DURING SKELETAL MYOBLAST DIFFERENTIATION

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by

YANNICK POULIOT

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Please refer to Page iv for a statement of my contributions to co-authored publications.

ABSTRACT

Using a polyclonal anti-cadherin serum, a cadherin was detected in the rat L_6 myoblasts cell line. Levels of this cadherin peaked when myoblasts began to fuse together in vitro. BUdR, an inhibitor of the program of terminal myogenic differentiation, severely lowered levels of this cadherin. Blockade by anti-cadherin immunoglobulins inhibited myoblast fusion. These data suggest that this cadherin is regulated by the program of terminal differentiation and that it plays a role in myoblast fusion.

Two cadherins are now known to be expressed in mammalian myoblasts: N-cadherin and M-cadherin. Using Northern analysis, both L_6 and mouse C2 myoblasts were shown to express M-cadherin. However, differentiating L_6 cells did not express detectable levels of N-cadherin, although N-cadherin is expressed in both C2 and rat primary myoblasts. Thus, L_6 myoblasts are expression mutants for N-cadherin, indicating that this cadherin is not essential for the differentiation and fusion of these myoblasts. In contrast, M-cadherin transcripts were detected in all myoblast cell lines studied, and were developmentally regulated during the differentiation of L_6 myoblasts in vitro. The pattern of regulation was similar to that of muscle-specific genes, with the exception that M-cadherin levels decreased once the cells had differentiated. In vivo, M-cadherin transcripts were restricted to embryonic skeletal muscle and were undetectable in adult tissues. Furthermore, BUdR down-regulated the expression of M-cadherin in both C2 and L_6 myoblasts, suggesting that M-cadherin is regulated by the myogenic differentiation program.

Lastly, (1) I discovered that cadherins and influenza strain A hemagglutinin share sequence similarity, (2) described the first phylogenetic analysis of the cadherin superfamily and (3) contributed in the discovery of the cadherin cell adhesion recognition site.

RÉSUMÉ

À l'aide d'un anticorps polyclonal anti-cadhérine, j'ai détecté une protéine immunoréactive exprimée par les myoblastes squelettiques de la lignée cellulaire L_6. Le niveau maximum de cette cadhérine fut observé lorsque les myoblastes commencaient à fusionner. Ce niveau fut sévèrement diminué par le BUdR (un inhibiteur de la differenciation myoblastique). Les immunoglobulines anti-cadhérine inhibèrent la fusion des myoblastes. Ces données suggèrent que cette cadhérine est contrôlée par le programme de différenciation terminale et qu'elle joue un rôle dans la fusion des myoblastes.

Deux cadhérines sont maintenant connues pour être exprimées par les myoblastes squelettiques de mammifères: la N-cadhérine et la M-cadhérine. En utilisant la technique d'hybridization "Northern", j'ai démontré la présence de transcripts de M-cadhérine chez les myoblastes L_6 et C2 (provennant de la souris). Cependant, les transcripts de N-cadhérine ne sont pas détectables chez les myoblastes L_6 capables de différenciation, contrairement avec les cellules C2 et lées myoblastes de rat. Les myoblastes L_6 sont apparament des mutants incapables d'exprimer cette cadhérine. La N-cadhérine n'est donc pas nécessaire pour la fusion et la différenciation de ces myoblastes. Par contre, les transcripts de M-cadhérine furent détectés chez tous les myoblastes testés, et son niveau est contrôlé durant la différenciation in vitro des myoblastes L_6. La régulation de son expression est similaire à celle des gènes squelettiques, à l'exception de la diminution du niveau des transcripts après la différenciation. In vivo, les transcripts de M-cadhérine sont détectés uniquement dans le muscle squelettique embryonnaire, et ne furent pas détectés parmi les tissus adultes du rat. La BUdR diminue profondément l'expression de la M-cadhérine aussi bien chez les myoblastes L_6 que C2. Ces données suggèrent que la M-cadhérine est contrôlée par le programme de différenciation terminale myogénique.

En dernier lieu, j'ai découvert (1) une similarité de séquence entre les cadhérines et l'hémagglutinine de l'influenza de type A, (2) décrit la première analyse phylogénétique de la superfamille des cadhérines et (3) contribué à la découverte du site d'adhésion intercellulaire des cadhérines.

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I would also like to thank Drs. Blaschuk, Clark, Hastings and Nishioka for serving on my advisory committee, and for all their helpful discussions throughout this project. Special thanks to the faculty of the MNI, particularly the members of the Neuromuscular Research Group, as well as to Mr. C. Guérin, Ms. J. Papillion and Mrs. Agnes Mwase.

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During my Ph.D. training I was the recipient of a graduate fellowship from Fonds de la Recherche en Santé du Québec. Furthermore, I received bursaries to attend the 1991 Molecular Evolution workshop at the Woods Hole Molecular Biology Laboratory, as well as the 1992 Mathematical Sciences in Genomic Analysis Workshop of the Societal Institute of the Mathematical Sciences. I was also a visiting scientist in the laboratory of Temple F. Smith at Harvard University. Research funds for this project were provided by the Medical Research Council of Canada and the Muscular Dystrophy Association of Canada to Dr. Holland.

For co-authored publications, the extent of my contribution is listed below:

Chapter 3: I performed assays to measure the effect of synthetic peptides on the plasma membrane fusion and aggregation of L_6 myoblasts and performed sequence analysis work.

Chapter 4: I originated the concept, defined the strategy, ran the database searches and evaluated the results.

Chapter 5: I performed the cellular assays (including micrography), contributed to the immunofluorescence experiments, prepared protein extracts and densitometric scans.

Chapter 6: All experiments, except the dissection and subsequent RNA extraction from embryonic skeletal muscle, were performed by me.

Most of the results presented here were published in the following articles:

Blaschuk, O. W., Pouliot, Y. & Holland, P. C. (1990) Identification of a conserved region common to cadherins and influenza strain A hemagglutinins. Journal of Molecular Biology, **211**:679-682.

Blaschuk, O. W., Sullivan, R., David, S. & Pouliot, Y. (1990) Identification of a cadherin cell adhesion recognition site. Developmental Biology, **139**:227-229.

Pouliot, Y., Holland, P. C. & Blaschuk, O. W. (1990) Developmental regulation of a cadherin during the differentiation of skeletal myoblasts.

Developmental Biology, **141**:292-298.

Pouliot, Y. (1992) Phylogenetic analysis of the cadherin superfamily. BioEssays, **14**:743-748.

In addition, I also published the following papers on topics unrelated to my thesis work:

Bengio, Y. & Pouliot, Y. (1990) Efficient recognition of immunoglobulin domains from amino acid sequences using a neural network. Computer Applications in the Biosciences, **6**:319-324.

Bengio, Y., Pouliot, Y., Bengio, S. & Agin, P. (1990) A neural network to detect homologies in proteins. In: Advances in Neural Information Processing Systems 2, Touretzky, D. S. (ed.), Morgan Kaufmann, San Mateo, CA, pp. 423-430.

Cashman, N. R. & Pouliot, Y. (1990) EBV Ig-Like Domains. Nature, **343**:319.

Karpati, G., Pouliot, Y., Zubrzycka-Gaarn, Carpenter, S., Ray, P. N., Worton, R. G. & Holland, P. (1989) Dystrophin is expressed in *mdx* skeletal muscle fibers after normal myoblast implantation. American Journal of Pathology, **135**:27-32.

Karpati, G., Pouliot, Y., Carpenter, S. & Holland, P. C. (1989) Implantation of nondystrophic allogenic myoblasts into dystrophic muscles of *Mdx* mice produces "mosaic" fibers of normal microscopic phenotype. In: Cellular and Molecular Biology of Muscle Development, Kedes, L. & Stockdale, F., (eds), UCLA Symposia on Molecular and Cellular Biology, New Series, Volume 93, Alan R. Liss, New York, NY, pp. 973-985.

Karpati, G., Pouliot, Y. & Carpenter, S. (1988) Expression of immunoreactive major histocompatibility complex products in human skeletal muscles. Annals of Neurology, **23**:64-73.

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LIST OF TABLES

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LIST OF ABBREVIATIONS

bp: base-pair

BUdR: 5-bromo-2'-deoxyuridine

CAR: cell adhesion recognition site

CAM: cell adhesion molecule

CD: calcium-dependent

CI: calcium-independent

CNS: central nervous system

DMEM: Dulbecco's Modified Essential Medium

DTT: dithiothreitol

EC: extracellular

EDTA: ethylenediaminetetraacetic acid

EGTA: (ethylene-dioxy)diethylenedinitrilotretaacetic acid

FBS: fetal bovine serum

FGF: fibroblast growth factor

GAPDH: glyceraldehyde-3-phosphate-dehydrogenase

HA: hemagglutinin

HEPES: N-2-hydroxyethylpiperaine-N'-ethanesulphonic acid

HS: horse serum

ITS: Insulin, transferrin, selenium

kb: kilobase

kDa: kilo dalton

L-CAM: liver cell adhesion molecule

mIU: milli international unit

N-CAM: neural cell adhesion molecule

NGF: nerve growth factor

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

RT-PCR: reverse transcriptase/polymerase chain reaction

SDS: sodium dodecyl sulfate

SSPE: sodium, phosphate, EDTA

TBS: Tris buffered saline

TE: Tris-EDTA

1.0 INTRODUCTION

1.1 Preamble

This thesis describes my research accomplished in the pursuit of the Ph.D. degree in the laboratory of Paul C. Holland at McGill University (Montreal, Canada). It describes how cadherins are relevant to the process of myoblast fusion and how one particular cadherin, M-cadherin, appears to be restricted to skeletal muscle and how it may regulate myoblast differentiation. The thesis is assembled from four published papers and one submitted paper. The first paper reviews the structural features of members of the cadherin superfamily and describes the phylogenetic relationships between its members. The second paper relates the discovery of the cell adhesion recognition site of cadherins. On the same topic, the third paper describes the sequence conservation between this site and a region of the influenza strain A hemagglutinin. The fourth paper describes the immunological detection of a cadherin which is necessary for the plasma membrane fusion of L_6 line of rat skeletal myoblasts. Finally, the appendices will present relevant work which was not included in those publications.

While I was characterizing this cadherin, Donalies et al. (1991) reported the discovery of a M-cadherin, a novel member of the cadherin family expressed in immortalized mouse C2 myoblasts. This protein met many of the postulates I had established regarding a putative muscle-specific cadherin (see below). Although Donalies et al. demonstrated that M-cadherin was regulated during the differentiation of mouse C2 myoblasts, they did not provide evidence regarding its tissue distribution

or whether it is present in L_6 myoblasts. I thus proceeded to prove that the M-cadherin message and protein are present in L_6 myoblasts, that the expression of M-cadherin mRNA is restricted to developing embryonic skeletal muscles and that M-cadherin expression is regulated during myogenesis in vitro and in vivo. These results are described in the fifth chapter (paper submitted). In order for the reader to fully understand the conclusions presented herein, I will first provide a perspective on research in the field of myogenesis and cell adhesion.

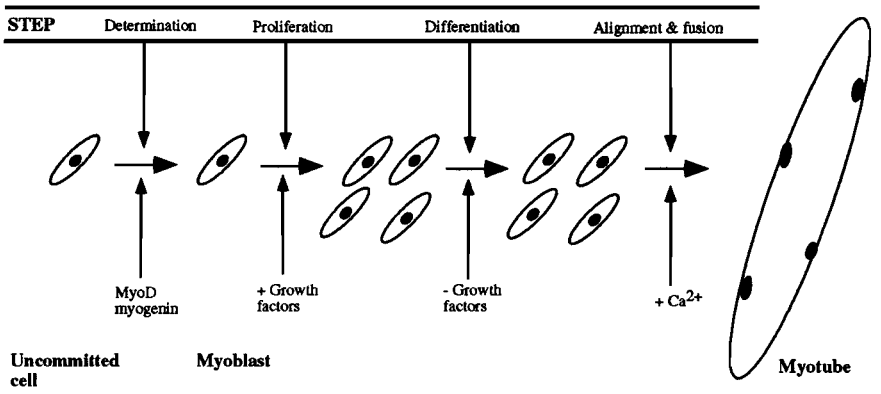
1.2 Myogenesis in vitro and in vivo

Skeletal muscle cells account for over a quarter of the mass of the human body and most of its energy expenditure. They implement the posture and motion of the body through their attachment to ligaments which connect them with the bones of the skeleton. These functions require an extraordinarily complex cellular structure, possibly the most complex of any cell type. Skeletal myogenesis has been studied extensively both in vitro and in vivo in a wide range of vertebrate and invertebrate species. Here I will largely restrict myself to an overview of results obtained in avian and mammalian species. Mature individual muscle cells, termed "myofibers", can be as long as the length of the muscle of which they are part, and range from 10 to 100 μm in diameters. Most myofibers are formed during embryogenesis following the plasma membrane fusion of large numbers of muscle precursor cells, termed "myoblasts", to form myotubes which then become innervated and differentiate further (for review, see Schneider & Olson, 1988). This process, termed terminal differentiation, is irreversible

and is accompanied by the permanent withdrawal of myotubes from further cell division. The principal steps of this process are outlined in Fig. I:1. In mammals, myoblast fusion continues some time after parturition. Myoblasts themselves are formed early in development (reviewed in Ott et al., 1990). For the purpose of this thesis I will define a myoblast as a cell which expresses at least some members of the set of muscle determination genes which are part of the helix-loop-helix (HLH) family (e.g., myogenin, Wright et al., 1989; and MyoD1, Tapscott et al., 1989), and which confer myogenicity when expressed in certain fibroblastic cells (reviewed in Wright, 1992). Transcription of these genes is inhibited by the thymidine analog, 5-bromo-2'-deoxyuridine (BUdR), thus inhibiting terminal differentiation (cf., Tapscott et al., 1989). In the mouse, two members of the HLH family, myogenin and myf-5, can be detected at ~E8 in cells of the myotomes (Sassoon et al., 1989; Ott et al., 1991; reviewed in Sassoon, 1993). As indicated above, myofibers are formed by the fusion of individual myoblasts. This process occurs in two "waves", in which distinct populations of myoblasts proceed to form muscle in discrete steps.

Fig. I:1. Overview of the main features of skeletal myogenesis

The process of skeletal myogenesis as understood from in vitro and in vivo studies is outlined. Myoblasts arise from stem cells present in the embryonic somite, which differentiate to become myogenic cells following the induction of muscle determination genes such as MyoD and myogenin. Once committed, these cells proliferate in response to peptidic growth factors such as FGF and TGF-β. A reduction in the concentration of these growth factors induces withdrawal from the cell cycle, concomitant with the induction of muscle-specific genes such as the muscle isoforms of creatine kinase and myosin. In parallel with this induction, myoblasts align and fuse with each other to form a syncytial cell capable of contraction, the myotube. This process is dependent upon calcium for alignment and for fusion per se. Steps subsequent to myotube formation, such as innervation and fasciculation, are not depicted. In the mouse, the first wave occurs between E14 and E16 and forms the primary myotubes (Ontell et al., 1988). These myotubes form the scaffold which the second wave of myoblasts will use to form secondary myotubes. This secondary wave accounts for most of the muscle mass and, in the mouse, occurs between E18 and a few days after birth (Ontell et al., 1988).


STEP
Determination
Proliferation
Differentiation
Alignment & fusion
MyoD
myogenin
+ Growth
factors
- Growth
factors
+ Ca2+
Uncommitted
cell
Myoblast
Myotube

1.21 Role of cell adhesion in myogenesis

The process of myoblast fusion can be divided into distinct stages: withdrawal from the cell cycle, induction of the set of muscle-specific genes found in mature muscle, alignment and plasma membrane fusion (reviewed in Knudsen, 1990a; see Fig I:1). Although the coordinate induction of the muscle-specific forms of genes such as creatine kinase (Delain et al., 1973; Perriard, 1979) and myosin (Devlin & Emerson, 1978; Paterson, B. & Strohman, R. 1972) is coincident with plasma membrane fusion, fusion is not required for induction of these genes. This was demonstrated by showing that chicken myoblasts cultured in medium depleted of calcium by chelation by EGTA can still differentiate although they cannot fuse (Patterson & Strohman, 1972; Holland & Maclennan, 1976). The principal criteria for myoblast differentiation are withdrawal from the cell cycle (Emerson & Beckner, 1975) and termination of DNA synthesis (Nadal-Ginard, 1978), along with the coordinate induction of the muscle-specific genes associated with the mature muscle phenotype. In vitro studies have shown that termination of cell division is dependent upon the concentration of specific growth factors such as TGF-β and FGF, such that high concentrations of these factors promote continued cell division (reviewed in Florini & Magri, 1989). The alignment and fusion steps can be further subdivided into three additional stages: cell-type recognition, cell-cell adhesion and membrane union (Fig. I:1). This thesis is concerned with the role of cell adhesion molecules in mediating the intercellular adhesive interactions which lead to the fusion of apposing myoblasts. Two types of intercellular adhesion systems have been observed in many cell types: a calcium-dependent (CD) system and a calcium-

independent (CI) system (cf. Gibralter & Turner, 1985). Both systems are present in myoblasts, and individual CAMs belonging to each class are involved in myogenic differentiation (see below). Because a substantial rise in calcium-dependent cell adhesion is observed as chicken myoblasts fuse (Knudsen & Horwitz, 1977), the CD adhesion system has long been thought to play an important role in bringing apposed cell membranes into close contact so as to form stable adhesive interactions leading to membrane fusion. In addition to cadherins, other CAMS such as integrins and neural cell adhesion molecules (N-CAMs) are also expressed in differentiating myoblasts (reviewed in Knudsen, 1990a). These belong to the CI cell adhesion system. Many alternative splice products of the N-CAM gene have been identified in different cell types and several forms are developmentally regulated during myogenesis in vitro (Moore et al., 1987). In addition, a skeletal muscle-specific form characterized by the presence of an exon which is spliced only in skeletal myoblasts, has also been identified (Dickson et al., 1987). However, no function could be ascribed to this form of N-CAM. Similarly, while individual N-CAM species are developmentally regulated during myoblast differentiation and have been implicated in skeletal myogenesis (Knudsen et al., 1990b), subsequent analyses (Mege et al., 1992) suggest that N-CAM does not play a significant role in myoblast fusion. Dickson et al. (1990) used transfection experiments to show that a hundred-fold overexpression of exogenous N-cadherin mRNA over endogenous transcripts led to a two-fold increase in the extent of plasma membrane fusion of transfected C2 myoblasts. However, Mege et al. (1992) observed no effect of anti-N-CAM antibodies upon the differentiation of chicken

myoblasts, although N-cadherin antibodies were effective. These results are in agreement with those obtained earlier by Rutishauser et al. (1983), who found no effect of anti-N-CAM antibodies upon myoblast fusion.

There is good evidence that another family of CAMs, the integrins, are involved in the control of myogenic differentiation. Integrins are dimeric integral membrane proteins expressed in a wide array of cell types where they mediate cell-cell or cell-matrix adhesion (for review, Hynes, 1992). Their expression is spatio-temporally regulated. Two reports have demonstrated a direct role for integrins in myotube formation. Menko and Boettinger (1987) first demonstrated that cultivation of chick myoblasts in the presence of the CSAT antibody inhibits both myoblast differentiation and fusion. However, because this antibody is directed against the β_1 subunit, the identity of the α subunit is unclear. Furthermore, antibodies directed against either VLA-4 ($\alpha_4\beta_1$) or its counter-receptor, VCAM-1, inhibit myotube formation in vitro (Rosen et al., 1992). Lastly, the $\alpha_7\beta_1$ integrin is known to be tightly regulated during myoblast terminal differentiation, and has been shown to be susceptible to a variety of treatments which interfere with the myogenic differentiation program (Song et al., 1992). No direct evidence linking this integrin to myoblast differentiation has been described, however.

1.22 Cadherins: structure, expression, function

[Please note that the cadherin superfamily and the structural features of its members are reviewed comprehensively in Chapter 2, such that this section will focus

largely on points not covered in that review.]

Cadherins form a large family (cf. Table I:1) of membrane proteins, most of which range in size from ~120-135 kDa. The cadherin family itself is part of a superfamily of membrane proteins, which includes protocadherins (Suzuki et al., 1991) and the desmocollins/desmogleins (Holton et al., 1990; Goodwin et al., 1990, respectively; reviewed in Buxton & Magee, 1992). The adhesive activity of CAMs can be characterized as homotypic or heterotypic, depending on whether they mediate adhesion between identical molecules present on apposing cells (e.g., cadherins) or between a molecule and its receptor (e.g, certain integrins and fibronectin). In the case of cadherins, intercellular adhesion is homotypic and requires calcium. This was best demonstrated by transfecting mouse L cells, which do not exhibit calcium-dependent cell adhesion and do not express cadherins, with either E-, P- or N-cadherin and then performing aggregation assays using stable transfectants (Nose et al., 1988; Miyatani et al., 1989). Using this assay it was demonstrated that cadherins preferentially interact with their own subtype, and that this interaction can be reversibly inhibited by EGTA. Although cadherins mediate homotypic cell adhesion, under some conditions they can also mediate heterotypic adhesive interactions, albeit with an affinity thought to be lower (Volk et al., 1987). Lastly, although most cadherins are integral membrane proteins, at least one member of the superfamily (T-cadherin) is linked to the outer leaflet of the plasma membrane by a glycosyl phosphatidylinositol linkage (Ranscht, B. & Dours-Zimmermann, 1991). As with many membrane proteins, glycosylation and phosphorylation have been demonstrated for certain cadherins (e.g., L-CAM,

Cunningham et al., 1984), and it is assumed that all cadherins are glycosylated and phosphorylated since the apparent molecular mass of individual cadherins as deduced from their migration under SDS-PAGE differs considerably from the molecular mass predicted from the conceptual translation of their respective cDNA.

Cadherins were first discovered by their resistance to proteolysis in the presence of calcium. Cells treated with trypsin in the presence of millimolar concentrations of calcium retained a calcium-dependent cell adhesion activity, whereas cells treated with trypsin in the absence of calcium lost this activity (Takeichi, 1977). This datum could be correlated with the disappearance of a band detectable by SDS-PAGE in total cell protein extracts of cells trypsinized in the presence of calcium but absent in cells trypsinized in the absence of calcium (Takeichi, 1977). This characteristic resistance of cadherins to trypsinization in the presence of calcium was used to screen various polyclonal and monoclonal antisera and led to the immunological identification of the three "classical" cadherins, referred to as E-, P- and N-cadherin (reviewed in Pouliot, 1992; Chapter 2). These designations refer to the tissues to which the expression of these cadherins was initially thought to be restricted, i.e., epithelial (E), placental (P) and neural (N) (reviewed in Takeichi, 1988).

Table I:1. Properties of cadherins

The basic properties of all cadherins which have been molecularly cloned and for which the sequence is publicly available are listed. For several cDNAs the complete coding sequence is not available. The table does not include "desmosomal" cadherins (reviewed in Buxton & Magee, 1992). These molecules, while members of the cadherin superfamily, are distinct from cadherins since they harbour a different C-terminal region and do not interact with the actin microfilament network.

Notes

[1] grouping criteria are defined in Pouliot, 1992.

[2] NcalCAM, A-CAM and N-cadherin are the same molecule

[3] B-cadherin is most likely encoded by the K-CAM gene (Sorkin et al., 1991)

[4] As part of this work, I have determined the partial sequence of rat M-cadherin cDNA

[5] H. sapiens expressed sequence tag (EST) EST05302 exhibits high sequence similarity to G. gallus T-cadherin (Adams et al., 1993)

Legend

Bt: B. taurus; Gg: G. gallus; Hs: H. sapiens; Mm: M. musculus; Rn: R. norvegicus; Xl: X. laevis; N/A: not available.

Cadherin subtype	Species	Group[1]	GenBank accession number	Reference	No./size of transcripts (kb)	Gene size/chromo assignment
Neural (N)[2]	Hs	I	M34064	Walsh et al., 1990	3 (5.2, 4.3, 4.0)	(18)
	Mm	I	M31131	Miyatani et al., 1989	3 (5.2, 4.3, 3.5)	> 200 kb (p18)
	Gg	I	X07277	Hatta et al., 1988	1 (4.3)	
	Bt	I	X53615	Liaw et al., 1990	3 (5, 4.1, 1.7)	
	Xl	I	X57675	Fujimori et al., 1990	1 (4.2)	
Epithelial (E)/uvomorulin	Hs	I	X12790	Mansouri et al., 1989	1 (4.5)	
	Mm	I	X06115	Nagafuchi et al., 1987	1 (4.5)	> 40 kb (8)
Placental (P)	Hs	I	X63629	Shimoyama et al., 1989	1 (3.2)	
	Mm	I	X06340	Nose et al., 1987	1 (3.2)	> 45 kb (8)
	Bt	I	X53614	Liaw et al., 1990	2 (3.7, 3.3)	
E- and P-like (EP)	Xl	I	X63720	Ginsberg et al., 1991	1 (3.5)	
Brain (B)[3]	Gg	I	X58518	Napolitano et al., 1991	1 (3)	15 kb
Retinal (R)	Hs	I	N/A	Suzuki et al., 1991	N/A	
	Mm	I	X69966	Hutton et al., 1993	3 (6.8, 3.5, 1.8)	(2)
	Gg	I	D00849	Inuzuka et al., 1991	3 (7.4, 5.4, 4.6)	
Liver cell adhesion molecule (L-CAM)	Gg	I	M1620 J04074 M22179	Gallin et al., 1987	1 (4.3)	10 kb <
XB	Xl	I or II	X63719	Herzberg et al., 1991	1 (3.9)	
Muscle (M)	Hs	N/A	N/A	N/A	N/A	(16q24.1-qter)
	Mm	II	M74541	Donalies et al., 1991	1 (3.4)	(8)
	Rn[4]	N/A	N/A	Pouliot et al., submitted	2 (3.7, 3.4)	
Kidney (K)	Rn	II	D25290	unpublished	N/A	
Truncated (T)	Gg	III	M81779	Ranscht et al., 1991	4 (10, 7.5, 4.6, 3.2)	
	Hs	N/A[5]	T07413	Adams et al., 1993	N/A	
Truncated 2 (T2)	Gg	III	S62757	Sacristán et al., 1993	4 (4.9, 3.3, 2.8, 2.3)	

Studies in different vertebrates, as well as more extensive studies in the species in which these cadherins were discovered, now indicate that these designations suggest an overly restrictive pattern of expression, and that cadherins are often expressed in several different tissues unrelated to the tissue where the subtype was initially discovered (see below). Individual subtypes were initially thought to be expressed exclusively in specific tissues. However, it was later determined that the same subtype can be expressed in different tissues for a given developmental stage. Particularly interesting is the lack of tissue specificity often observed when one compares the expression profiles of cadherins across species boundaries. This is most striking in the case of P-cadherin. While abundant in the mouse placenta and absent from other tissues (Nose, A. & Takeichi, M., 1986), P-cadherin is expressed at very low levels in human placenta (Shimoyama et al., 1989) and in B. taurus is easily detected in tissues other than placenta (Liaw et al., 1990).

The expression pattern of individual cadherins is spatially and developmentally regulated. Almost all tissues studied express at least one cadherin, and often more than one. Furthermore, the same cadherin subtype is often expressed by several tissues simultaneously. However, studies of chicken and frog embryos show that the segregation of cells from a germinal layer is characterized by an exclusive profile of expression which is distinct between cells of an organ and surrounding cells, and by a switch in the subtype of cadherin expressed (e.g., Hatta, K. & Takeichi, M., 1986; Hatta et al., 1986). For example, during the invagination of the lens placode in the chicken, cells which begin to express N-cadherin will stop expressing L-CAM (Hatta

& Takeichi, 1986). The establishment of such exclusive expression patterns has been observed in several morphogenetic phenomena, which, in the chicken, include gastrulation, neurulation, as well as lens formation (Hatta et al., 1987). These observations suggest that the level of expression, cadherin subtype and the proper localization of cadherins play important roles in different stages of embryogenesis. The hypothesis that differential cadherin expression can lead to cell sorting has been confirmed in vitro in several ways. In one experiment using the L cell/expression vector transfection model described above, the co-cultivation in vitro of two populations of mouse cell lines expressing either N- or E- cadherin led to the formation of homogeneous groups composed of cells expressing one or the other cadherin (Miyatani et al., 1989). The importance of cadherins in morphogenesis in vivo has been demonstrated using frog embryos, in which expression of N-cadherin expression preceded the formation of the neural plate and tube. In experiments where N-cadherin mRNA was micro-injected into ectodermal cells prior to the neural induction so as to induce premature expression, dramatic morphological defects are observed, indicating that the proper timing of N-cadherin expression is critical for normal embryogenesis (Detrick et al., 1990; Fujimori et al., 1990). In contrast, the same experiment performed with N-CAM mRNA had little effect (Detrick et al., 1990).

As with integrins, cadherins exhibit specific subcellular localization. For example, in highly polarized cells such as epithelial cells, E-cadherin molecules are restricted to the baso-lateral membrane and are not found in the apical side of the cell

(Hatta & Takeichi, 1986; Nose & Takeichi, 1986; Hatta et al., 1987). The known cadherins appear to be concentrated in a class of cell-cell junctions alternatively termed adherens junction, belt desmosome, zonula adherens or intermediate junction (Boller et al., 1985; Volk, T. & Geiger, B., 1986a,b). For simplicity I will use the term adherens junction. Adherens junctions are focal points for cortical actin bundles, to which cadherins colocalize (Boller et al., 1985; Volk & Geiger, 1986a,b; Hirano et al., 1987). This was demonstrated by treating cells with the non-ionic detergent NP-40. In such cells, most of the cadherin molecules remain insoluble and are localized to adherens junctions via the attachment of the C-terminal cytoplasmic domain to the microfilament network (Hirano et al., 1987). Interestingly, cadherins are easily extracted from cells following removal of calcium by treatment of cells with EGTA, therefore disrupting cadherin-mediated cell adhesion (Hirano et al., 1987). The importance of calcium binding sites for the functional activity of cadherin has been demonstrated in experiments where a single amino acid conservative substitution in one of the calcium-binding sites of cadherin is sufficient to abolish both the binding of calcium and cell adhesion (Ozawa et al., 1990). However, the exact sequence of events required for localization of cadherins to adherens junctions remains unknown.

The role of the C-terminal region of cadherin in cell adhesion has been studied extensively by deletion analysis of the cytoplasmic domain of cadherin (Nagafuchi & Takeichi, 1988; Ozawa et al., 1988; reviewed in Kemler & Ozawa, 1989). Such deletion mutants are incapable of mediating calcium-dependent cell adhesion, even though the truncated cadherins are expressed and translocated properly (Nagafuchi &

Takeichi, 1988). Furthermore, they do not interact with catenins (see below) and do not localize to adherens junctions. These studies clearly demonstrated the importance of the cytoplasmic domain in controlling cadherin-mediated cell adhesion, and led to the discovery of the catenins.

The catenins were initially noticed as co-precipitants in cadherin immunoprecipitation experiments. Three catenins, α, β and γ, have been identified so far (Nagafuchi & Takeichi, 1988; Ozawa et al., 1989; McCrea & Gumbiner, 1990). A variant of α-catenin, termed neural α-catenin, has also been identified and is mainly expressed in neural tissues (Hirano et al., 1992). Catenins form a family of unrelated proteins which share their capacity to interact with the cadherin cytoplasmic domain. Interestingly, these molecules are homologs of previously characterized cytoskeletal-associated molecules. α-Catenin (Mr 102 kDa) is related to vinculin (Herrenknecht et al., 1991; Nagafuchi et al., 1991) while β-catenin (Mr 92-98 kDa) is related to the Drosophila armadillo protein as well as to plakoglobin (McCrea et al., 1991). Regarding γ-catenin (Mr 83 kDa), it is unclear if it is plakoglobin or a related molecule (Knudsen & Wheelock, 1992). Catenins appear to modulate cadherin-mediated cell-cell adhesion, since tyrosine phosphorylation by $p60^{v\text{-}src}$ of the N-cadherin/catenin complex suppresses calcium-dependent cell aggregation without affecting the levels of cell surface cadherin (Matsuyoshi et al., 1992).

1.23 Discovery of the cadherin cell adhesion recognition site

Apart from the cytoplasmic tail, only the first extracellular (EC 1) domain of

cadherins has been shown to have functional activity. This had long been suspected since almost all monoclonal antibodies capable of interfering with cadherin-mediated cell adhesion bind to an epitope in that domain (cf. Fig 8, Nose et al., 1990). Two experimental strategies, one based upon the co-cultivation of cells with candidate synthetic peptides (Blaschuk et al., 1990a; Chapter 3) and the other relying upon site-directed mutagenesis, converged on the same region of the EC1 domain (Nose et al., 1990). This region is characterized by the presence of a tripeptide which is highly, though not universally, conserved in cadherins, HAV. These experiments are reviewed in Pouliot, 1992 (Chapter 2) and led to the identification of the cadherin cell adhesion recognition site (CAR site). Specific synthetic peptides containing the CAR site sequence can inhibit cadherin-mediated adhesion in several developmental processes (Blaschuk et al., 1990a; Doherty et al., 1991a; Mege et al., 1992). In parallel with these experiments, I also analyzed the contents of the GenBank sequence database to determine whether other proteins might contain a sequence similar to that of the cadherin CAR site. This led to the discovery of a highly significant sequence similarity between a functionally important region of the influenza strain A hemagglutinin and the CAR site region of cadherins (Chapter 4). This paper was the first demonstration of a sequence similarity between cadherins and another class of proteins.

1.24 Involvement of cadherins in disease states

In addition to their role in morphogenesis, cadherins are also thought to be involved in pathogenic phenomena dependent upon cell adhesion, such as neoplastic

invasion and metastasis (Behrens et al., 1989; Hashimoto et al., 1989; Frixen et al., 1991; reviewed in Takeichi, 1993). It is postulated that transformed cells which have lost cadherin-dependent cell adhesion are more likely to become invasive than cells which have retained this activity. In the latter case, such tumour cells are thought to be more likely to remain benign, that is, remaining localized to an organ system and not penetrating organ boundaries. So far, this postulate has been shown to be largely correct for carcinomas, where tumours with low levels of E-cadherin expression are more likely to be invasive than tumours with high levels of E-cadherin (cf. Takeichi, 1993). In cases where the levels of E-cadherin are normal and the protein is normally translocated to the cell surface, an absence of α-catenin can nonetheless lead to low cadherin-dependent cell adhesion, again resulting in enhanced invasiveness (Shimoyama et al., 1992).

1.25 Molecular genetics of cadherins

Recently, several cadherins have been mapped to individual chromosomes and their genes isolated and characterized (see Table I:1). Cadherins are almost always encoded by a single gene, the sole exception being the presence of N-cadherin pseudoalleles which are both expressed in Xenopus (Simonneau et al., 1992). Cadherin pseudogenes have not been reported. While multiple RNA species can be detected by Northern blotting for some cadherins (e.g., N-cadherin, Nose et al., 1989), alternatively spliced transcripts have not been isolated.

Cadherin genes can be very large due to the large size of the first and, in the

case of N-cadherin, second intron (Miyatani et al., 1992). Thus, cadherin gene sizes range from less than 10 kb to over 200 kb (Table I:1). Their exonic structure is highly conserved across species. In some cases, such as for the L-CAM and K-CAM (B-cadherin) genes, cadherins genes are tandemly arranged, suggesting local chromosomal duplication and independent molecular evolution thereafter (Sorkin et al., 1991). However, other cadherins remain as yet unlinked to other cadherins. This is most striking in the case of N- and R-cadherins. Although N-cadherin is highly similar to R-cadherin, it is localized to mouse chromosome 18, whereas R-cadherin is localized to chromosome 2 (Matsunami et al., 1993).

The study of the 5' flanking sequences of the P- and E-cadherin genes (Faraldo & Cano, 1993; Behrens et al., 1991 and Ringwald et al., 1991, respectively) has revealed little beyond the absence of TATA boxes in both of these promoters and the existence of an E-pal site in the E-cadherin promoter, a regulatory element active in epithelial cells.

1.26 Signal transduction by cadherins

In addition to mediating intercellular adhesion, the hypothesis that cadherins might also mediate signal transduction has been directly addressed in the PC12 adrenal pheochromocytoma cell line. This cell line (which expresses N-cadherin) has been very useful in studies of nerve growth factor since PC12 cells resemble late neural precursor cells but can be induced to differentiate to a neuronal phenotype when treated with nerve growth factor (NGF) or FGF. This is most strikingly observed in the "sprouting"

expression vector induces neuronal differentiation which is inhibited by anti-N-cadherin antibodies (Doherty et al., 1991b). Intracellular Ca^{2+} fluxes appear to mediate this effect since it could be inhibited by drugs such as pertussis toxin, verapamil and diltiazem, which inhibit muscarinic receptor-induced Ca^{2+}-influxes. However, the induction of differentiation is distinct from that resulting from treatment with NGF, most notably in not being dependent upon gene transcription (Doherty et al., 1991b). Furthermore, these authors do not provide a mechanism to directly link N-cadherin with the cellular response they observed.

Although the hypothesis of signal transduction was not directly assessed, Mege et al. (1992) observed that treatment with either anti-N-cadherin antibodies or a synthetic peptide containing the HAV sequence inhibits chicken skeletal myoblast differentiation, suggesting that N-cadherin may also mediate signal transduction in these cells.

Thus, while no direct mechanism of cadherin-mediated signal transduction has been proposed to account for these effects, cadherins are believed to be capable of conveying signals with profound effects upon cellular phenotype.

1.27 Cadherins in myogenesis: A role for M-cadherin in myoblast differentiation

My work and that of others demonstrated that cadherins are important in the process leading to the plasma membrane fusion of skeletal myoblasts (Mege et al., 1992; Knudsen et al., 1990c; Pouliot et al., 1990a, Chapter 5) and in the control of myogenic differentiation (Mege et al., 1992). The fusion of apposing plasma membranes is a crucial step in myogenesis since muscle cells must become multinucleated to generate the mechanical traction to move bones. Cell adhesion between apposing myoblasts should

myogenic differentiation (Mege et al., 1992). The fusion of apposing plasma membranes is a crucial step in myogenesis since muscle cells must become multinucleated to generate the mechanical traction to move bones. Cell adhesion between apposing myoblasts should therefore constitute an important step in bringing cells in close apposition to achieve membrane fusion. This view has prompted the search for cell adhesion proteins involved in myogenesis, with the most notable result being the discovery of integrins (Horwitz et al., 1985). While integrins have been shown to be involved in the control of myoblast differentiation in vitro (Menko & Boettinger, 1987), I and others (Pouliot et al., 1990a, Chapter 5; Knudsen et al., 1990b, respectively) postulated that cadherins might also be involved in this process, especially since the molecules responsible for the CD component of the cell adhesion activity observed in differentiating myoblasts remained to be identified. Two roles could be postulated for cadherins in myogenesis. The first role postulated that cadherins might be involved in providing a mechanism of intercellular adhesion so as to permit close membrane apposition and facilitate membrane fusion. Alternatively, as described above, cadherins might mediate a form of signalling similar to that observed with certain integrins. Of course, these functions need not be mutually exclusive.

When I began studying the role of cell adhesion in myoblast differentiation, I surmised that if a cadherin were implied in this process, it would likely be an unknown subtype possessing a CAR site devoid of the HAV tripeptide found in previously characterized cadherins. I further surmised that this cadherin would also probably be expressed solely in skeletal muscle and would be under the control of the myogenic

differentiation program so as to be expressed coordinately with muscle-specific genes. The discovery of M-cadherin by Donalies et al. (1991) (see below) substantiated these postulates. The primary structure of M-cadherin is devoid of the HAV tripeptide, which has been identified as the cadherin CAR site and which is replaced by the sequence FAL in M-cadherin. This substitution suggests that M-cadherin may be much more restricted in mediating cadherin-specific cell adhesion than other cadherins, preventing adhesion between M-cadherin and different cadherin subtypes since most cadherins harbour the HAV tripeptide. The expression of M-cadherin in C2 myoblasts and its developmental regulation in these cells suggested that it might play the specific role in myoblast differentiation which I had ascribed to an as-yet unidentified cadherin. My work later indicated that the M-cadherin message is detectable exclusively in developing skeletal muscle. On this basis I focussed my studies upon M-cadherin in order to determine its role in L_6 differentiation and fusion in vitro.

1.3 Objectives of thesis

1.31. Studies of cadherin structure

- To identify the CAR site of cadherins
- To determine whether other proteins harbour this site
- To determine the phylogenetic relationships of cadherins based upon the domain which harbours the cadherin CAR site

1.32. Identification and role of a cadherin in L_6 myoblasts

- To determine whether a cadherin can be detected immunologically in L_6 myoblasts
- To determine whether this cadherin is developmentally regulated during myoblast differentiation in vitro
- To determine if this cadherin is involved in myoblast fusion

• To determine the tissue-specificity of M-cadherin expression
• To determine whether M-cadherin is regulated during myoblast differentiation in vitro and in vivo, and comparing this pattern of regulation with that of $\alpha_5\beta_1$other muscle genes
• To determine whether M-cadherin might be a novel member of the set of muscle-specific genes
• To evaluate whether M-cadherin might play a role in myoblast fusion

1.4 Claims to originality

1.41 Discovery of the cadherin CAR site

In an attempt to identify reagents which might interfere with cadherin function, I studied the effect of synthetic peptides containing the CAR site upon myoblast fusion. Results obtained in other systems are presented here:

• The first demonstration of the cadherin CAR site using perturbation studies of neurite extension and blastula compaction by synthetic peptides containing the HAV sequence.
• The discovery of the sequence similarity between a functionally active region of influenza strain A hemagglutinin with the cadherin CAR site. This was the first report of a sequence similarity observed between the cadherins and another family of proteins.

• I reported the first computerized phylogenetic analysis of the cadherin superfamily, relating members of the cadherin superfamily to one another based upon the sequence of the EC1 domain.

1.42 Developmental regulation and role of cadherin in L_6 myoblasts

My M.Sc. thesis demonstrated that L_6 myoblasts exhibit calcium-dependent cell adhesion, and that this activity was probably mediated by a cadherin. As part of my Ph.D. research I proceeded to identify this cadherin.

• I reported the first detection, using immunological means, of a cadherin in L_6 myoblasts.

• This cadherin was immunolocalized in L_6 myoblasts and myotubes.

• Levels of this cadherin were also shown to be increased prior to myoblast fusion in vitro.

• I reported the susceptibility of levels of this cadherin to the differentiation inhibitor, BUdR. This is so far the only cadherin susceptible to this agent, and suggests that it is part of the myogenic differentiation program.

• I reported the first demonstration of inhibition of myoblast fusion by anti-cadherin immunoglobulin blockade.

1.43 Developmental regulation of M-cadherin mRNA and protein in vitro and in vivo

While investigating the identity of the cadherin expressed in L_6 myoblasts, M-cadherin was isolated. This cadherin may be the subtype I had been studying in L_6 cells. The immunological results I obtained in L_6 cells mirrored those I obtained with M-cadherin. A comparative characterization of M-cadherin N-cadherin expression in vitro and in vivo followed.

• I showed that M-cadherin, but not N-cadherin, is expressed in L_6 cells. This defines L_6 as an ideal system to study M-cadherin in isolation from N-cadherin.

• I demonstrated that M-cadherin is expressed in all myoblast cell lines studied.

• I demonstrated that M-cadherin is probably a novel skeletal muscle-specific gene:

1. M-cadherin expression is restricted to developing embryonic skeletal muscle
2. M-cadherin mRNA and protein are regulated during myoblast differentiation in vitro and in vivo. However, while M-cadherin is induced simultaneously with classical muscle-specific genes, it is down-regulated shortly after birth.
3. M-cadherin mRNA and protein are specifically down-regulated by BUdR.

• I demonstrated that cultivation of L_6 myoblasts with an M-cadherin antisense oligonucleotide inhibits myoblast fusion

2.0 -PHYLOGENETIC ANALYSIS OF THE CADHERIN SUPERFAMILY[1]

FOREWORD

During my study of the role of cadherin in myoblast differentiation and fusion, several novel cadherins were described. As a result, a large number of cadherin sequences from different species became available, rendering possible a molecular phylogenetic analysis. This analysis is presented in Chapter 2. The goal of this study was to help guide my thinking about the structural features of the EC1 domain of cadherin, particularly in contrasting features of M-cadherin with those of other cadherins. I had postulated that a muscle-specific cadherin would be molecularly distinct from other cadherins so as to provide highly specific interactions. The tree presented in Fig. II:2 indeed indicates that M-cadherin has long been evolving independently from the other cadherins, and that it is distinct from them. Thus, although no studies have demonstrated that M-cadherin can mediate intercellular adhesion, if M-cadherin does mediate such adhesion the uniqueness of its primary structure should provide highly specific adhesive interactions such as those observed in skeletal myogenesis.

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[1] Published as: Pouliot, Y. BioEssays, **14**:743-748 (1992)

2.1 Summary

Cadherins are a multigene family of proteins which mediate homophilic calcium-dependent cell adhesion and are thought to play an important role in morphogenesis by mediating specific intercellular adhesion. Different lines of experimental evidence have recently indicated that the site responsible for mediating adhesive interactions is localized to the first extracellular domain of cadherin. Based upon an analysis of the sequence of this domain, I show that cadherins can be classified into three groups with distinct structural features. Furthermore, using this sequence information a phylogenetic tree relating the known cadherins was assembled. This is the first such tree to be published for the cadherins. One cadherin subtype, neural cadherin (N-cadherin), shows very little sequence divergence between species, whereas all other cadherin subtypes show more substantial divergence, suggesting that selective pressure upon this domain may be greater for N-cadherin than for other cadherins. Phylogenetic analysis also suggests that the gene duplications which established the main branches leading to the different cadherin subtypes occurred very early in their history. These duplications set the stage for the diversified superfamily we now observe.

Most cadherins are integral membrane glycoproteins of molecular mass ~120 kDa which mediate homophilic, calcium-dependent intercellular adhesion. Several subtypes have been identified. Individual subtypes are encoded by single-copy genes which are controlled in a tissue- and developmental stage-specific fashion (for reviews, see refs. 1-3).

Cadherins are composed of several distinct domains (Fig. II:1). Starting from the amino terminal and extending toward the carboxyl end, the extracellular portion (EC) of cadherins is composed of a domain of ~110 amino acids which is tandemly repeated four times. These domains are designated EC 1-4. Following EC 4 is a fifth extracellular domain (EC5) with no clear relationship with the previous four. A highly conserved cytoplasmic domain usually follows a transmembrane region. The EC 1 domain is the best conserved domain of the extracellular portion of the protein across cadherin subclasses[3] and between species. Because of the conservation of the protein and gene structures among the various cadherin subtypes, cadherins are thought to have been generated by complete gene duplications rather than by exon shuffling (see below).

2.2 Identification of the Cadherin Cell Adhesion Recognition Site

In the last two years, two experimental strategies have contributed to the assignment of the region mediating cell adhesion in cadherin to the EC1 domain. Using site-specific mutagenesis, Nose *et al.*[4] have shown that changing a single residue flanking a highly conserved tripeptide, HAV, results in the loss of the specificity of adhesion observed between individual cadherin subtypes. A second approach relied upon the co-culture of cells in the presence of synthetic peptides containing the HAV sequence[5]. In systems where intercellular adhesion is known to be cadherin-mediated, culturing cells in medium containing synthetic peptides encoding the HAV motif resulted in significant inhibition of cell adhesion[5]. These

experiments indicate that the region encompassing the HAV motif harbours the cadherin cell adhesion recognition (CAR) region. However, the exact boundaries of this region have yet to be defined.

The presence of the CAR site in the EC 1 domain explains the strong conservation of this domain, since selective pressure would be expected to impose restrictions upon changes in the tertiary structure of the domain mediating cell adhesion. It also substantiates results obtained from early experiments, in which monoclonal antibodies capable of disturbing cadherin-mediated cell adhesion nearly always recognized epitopes localized to the EC 1 domain (see Fig. 8, reference 4). However, the high degree of sequence conservation of the EC 1 domain initially raised the question of how subtype-specific adhesion could be obtained from a domain with a primary structure which is highly conserved across different cadherin subtypes, each with different adhesive specificities. This question was resolved by the demonstration that even though cadherins are highly conserved in the region encompassing the HAV motif, substituting a single residue immediately flanking the HAV is enough to destroy subtype-specificity[4]. These results also explain why some degree of heterophilic adhesion can occur between cadherins of different subtypes, such as between A-CAM (N-cadherin) and L-CAM[6].

The data outlined above apply only to 'classical cadherins', herein defined as L-CAM, E- and N-cadherin (most recently reviewed in ref. 1), as well as to B-, EP- and R-cadherin. With the discovery of many new cadherin subtypes in the last two years, some of these new cadherins have been found to harbour deviations in the

sequence of their putative CAR site, as well as other alterations in their primary structure. These deviations suggest that the initial concept of the cadherin family as a highly coherent group of sequences consistently exhibiting the same features is too restrictive. Instead, it has been suggested that cadherins form a superfamily [7] which can be defined as a collection of molecules assembled from the cadherin repeat, and which may exhibit substantial sequence or structural divergence from classical cadherins. These deviations can be used as criteria for further grouping into subfamilies. One possible classification scheme, used to classify the cadherins listed in Table II:1, follows.

1. Group I cadherins: classical cadherins.

In this category are placed all full-length cadherins which harbour the HAV site. Most cadherins identified to date fall into this category.

Categories which do not follow the definition of group I cadherins ('unconventional cadherins') are placed in two additional groups:

2. Group II cadherins: full-length cadherins which do not harbour an HAV site but which are otherwise very similar to group I cadherins. Among the published cadherin sequences, only M-cadherin falls within this group.

3. Group III cadherins: cadherins which are missing segments found in either group I or II cadherins and which do not necessarily harbour an HAV site. So far, T-cadherin is the sole member of this group.

In addition to significant overall structural and sequence similarities with the cadherins, the final determination of whether a candidate molecule should be included

in the cadherin superfamily should rest upon whether this molecule can mediate calcium-dependent cell adhesion following transfection into a cell lacking any cadherin. So far this has been done with N-[8], E-[9], P-[10], R-[11], and T-cadherin[12], as well as with L-CAM[13]. Another feature of the cadherins is the interaction of their cytoplasmic tail with the actin microfilament network[14]. With the exception of molecules clearly related to cadherins but lacking a cytoplasmic tail (e.g., T-cadherin), this feature can also be used in confirming the identity of the candidate molecule.

The discovery of T-cadherin[12] is perhaps the best indication that the definition of the cadherin subfamily based upon strict adherence to the features displayed in group I cadherins is unreasonable. First, T-cadherin lacks the HAV motif. Second, instead of being linked to the cytoskeleton via a cytoplasmic domain as were other cadherins previously identified, T-cadherin lacks the transmembrane and cytoplasmic segments[12]. Rather, T-cadherin is linked to the extracellular leaflet of the plasma membrane via a glycosyl phosphatidylinositol bond[12]. The cytoplasmic region of full-length cadherins had been thought previously to be required for cell adhesion since deletions introduced in this region usually destroy adhesive activity[15]. The cytoplasmic region is now known to interact with a group of intracellular proteins collectively referred to as catenins[16,17] which mediate its interaction with the actin microfilament network. T-cadherin was therefore a surprising discovery, given the importance of the cytoplasmic domain in cadherin-mediated cell adhesion.

Table II:1. Cadherin sequences published to date

Cadherin sequences published to date[1]

Cadherin subtype	Species	Group	Sequence surrounding putative CAR site	GENBANK/EMBL accession number	Refs.
Neural (N)[2]	Human	I	ARFHLGAHAVDINGNQV	M34064	29
	Mouse	I	ARFHLRAHAVDINGNQV	M31131	8
	Chicken	I	ASFHLRAHAVDVNGNQV	X07277	3
	Bovine	I	ARFHLRAHAVDINGNQV	X53615	30
	Xenopus[3]	I	ANFHLRAHAVDVNGNQV	unavailable	19
Epithelial (E)/ uvomoruli	Human	I	ATYTLFSHAVSSNGNAV	X12790	31
	Mouse	I	AKYILYSHAVSSNGEAV	X06115	9
Placental (P)	Human	I	AKYELFGHAVSENGASV	X63629	32
	Mouse	I	VKYELYGHAVSENGASV	X06340	10
	Bovine	I	unavailable[4]	X53614	30
E- and P-like (EP)	Xenopus	I	DKYVLSSHAVSENGSPV	unavailable	33
Brain (B)	Chicken	I	NKYHLYSHAVSENGKPV	X58518	34
Retinal (R)	Chicken	I	ASYHLRAHAVDMNGNKV	D00849	11
Liver cell adhesion molecule (L-CAM)	Chicken	I	DRYTLLSHAVSASGQPV	M1620 J04074 M22179	13
Muscle (M)	Mouse	II	DRFRLRAFALDLGGSTL	M74541	35
Truncated (T)	Chicken	III	ANYELEVEVTDLSGKII	M81779	12

[1]Cadherins 8, 11, 12 and 13[7] are not included in this table since their full sequence has not yet been published.
[2]N-cadherin and A-CAM are the same molecule.
[3]A polymorphic allele of *Xenopus* N-cadherin exists[33]; only one allele was analyzed.
[4]The 5′ region encompassing the EC 1 domain of bovine P-cadherin has not been sequenced (C. Liaw, personal communication).

Also at variance with the structure of classical cadherins is the absence of an HAV motif in the putative CAR site of T-cadherin. The lack of this motif, or of a cytoplasmic domain, does not appear to hamper the adhesive activity of T-cadherin, since transfection of T-cadherin into a cell devoid of cadherin expression confers calcium-dependent aggregation[12]. Thus, the absence of these features does not constitute a sufficient criterion for exclusion from the cadherin superfamily. The absence of the HAV site in group II cadherins further suggests that the putative adhesive sites of these variant cadherins may have evolved so as to prevent the non-specific adhesive interactions observed between some of the cadherins harbouring the HAV site (see above). This is likely to be important in organs where many cadherins are known to be expressed, such as the central nervous system (CNS), where N-, T-, B- and R-cadherin are expressed, in addition to many other non-classical cadherins[7].

2.3 Phylogenetic Analysis of the Cadherin Superfamily

The relatively large number of cadherin subtypes available from different species now permits one to ask two phylogenetic questions: 'How are cadherins related to one another at the molecular level?' and 'What is their molecular evolutionary history?' To begin answering these questions, phylogenetic trees (Fig. II:2) were generated using the primary structure of the EC 1 domain of published cadherins (listed in Table II:1). This domain was chosen since it harbours all the sites known to be biologically active in the extracellular portion of the molecule. The sequence alignment used to generate the trees is provided in Fig. II:3. The trees were produced

using the maximum parsimony method and describe the evolutionary derivation of cadherins based upon the sequence of the EC 1 domain. The parsimony method generates trees based upon the smallest number of amino acid substitutions necessary to explain the derivation of a sequence from another, and constructs an evolutionary scenario (the tree) to fit this assumption. The branching order follows both the cadherin subtype and its species of origin. Because the EC 1 domain is well conserved among cadherin subtypes and species, definitive branch assignments can be difficult. Because of this, multiple, equally parsimonious trees are generated (in this case, 18 trees were produced). Nonetheless, a majority-rule consensus tree can be constructed. Such a consensus tree summarizes all trees by specifying the fraction of trees in which a branching assignment is followed. The consensus tree (Fig. II:2B) generated from the 18 equally parsimonious trees indicates that, in the majority of cases (>2/3), the branch assignments are identical. From the trees shown in Fig. II:2A and B, several conclusions can be drawn:

1. Cadherins distribute along three broad branches (Fig. II:2A,B). One branch leads to the N- and R-cadherins, one branch leads to both group II and III cadherins (e.g., M- and T-cadherin), and another branch leads to the P-, EP- and B-cadherins (Fig. II:2B). Assuming equal substitution rates during evolution (i.e. the molecular clock hypothesis), each of the gene duplications which founded these branches occurred very early in their evolutionary history.

2. The tree shown in Fig. II:2A indicates that the N-cadherins are much more conserved in the EC 1 domain than any other cadherin subtype so far isolated. This suggests that selection pressure upon this domain may be much greater for N-cadherins than for other cadherins. This high degree of sequence conservation may be related to their activity in the formation of the CNS. N-cadherin plays an important role in CNS formation since perturbation of N-cadherin during the development of the nervous system leads to severe morphological aberrations[18,19]. The requirements for CNS formation may be such that severe restrictions are imposed on the divergence of the EC 1 domain of N-cadherins. Other cadherins also expressed in the brain may yet exhibit these restrictions. However, the only other examples of cadherins expressed in the nervous system are all of chicken origin (e.g., B-, T-, R-),a and their counterparts in other species have not yet been isolated. The exception is cadherin 4, which has been identified tentatively as human R-cadherin[11].

3. Using the assumption of constant mutation rates, Fig. II:2A indicates that the R-cadherins have diverged from the N-cadherins recently and are most closely related to them. This is indicated by the smaller number of steps linking N- and R-cadherins together, compared to its next closest neighbour, P-cadherin (Fig. II:2A). That N- and R-cadherin share a relatively recent ancestor is not surprising, given that they have the highest overall sequence homology over their full length. Furthermore, these data are consistent with experimental evidence showing that R-cadherin can cross-adhere to N-cadherin in cell-sorting assays[11]. Interestingly, while R-cadherin is co-expressed with

N-cadherin in various neural and muscular tissues, the expression of R-cadherin is regulated differently from that of N-cadherin[20]. R-cadherin thus appears to be a homolog of N-cadherin which has diverged to acquire a developmental control, and presumably a function, distinct from that of N-cadherin.

4. Even with the small number of E- and P-cadherin sequences available, it is clear that these cadherins exhibit a much greater degree of sequence heterogeneity than do the N-cadherins (Fig. II:2A). This is particularly true for the large sequence divergence between mouse E-cadherin and chicken L-CAM, thought to be the chicken form of E-cadherin. This divergence suggests that perhaps L-CAM is not truly the chicken homolog of mouse E-cadherin. The isolation of additional E-cadherin sequences from other species is needed to form a broader perspective in order to resolve this point. This matter also raises questions such as whether some species may lack a specific cadherin, or whether species-specific cadherins exist.

5. While related to classical cadherins, M- and T-cadherin (groups II and III, respectively) have diverged very early from these and share only remote ancestors with them. This is consistent with the hypothesis that they may mediate highly specific intercellular adhesive interactions distinct from those of group I cadherins. Such specificity would be a logical requirement for tissues in which a mixture of group I, II and III cadherins are expressed. This is the case for skeletal muscle, in which N-, R-, M- and T-cadherin are co-expressed. In addition, M- and T-cadherin could also

mediate functions other than adhesive interactions, possibly reflecting the diversity and complexity of functions mediated by the tissues where they are expressed (neurons, skeletal muscle). Also of interest is the grouping of mouse M-cadherin with human desmosomal glycoprotein II (DG II)[21] (Fig. II:2A,B). Both DG I and DG II are members of a family of calcium-dependent cell adhesion molecules which are found in desmoglia and which share several structural features with cadherins (most recently reviewed in ref. 22). While this grouping of DG II and M-cadherin together suggests that DG II could be the human counterpart of mouse M-cadherin, this is probably not the case since the sequence of the human DG II EC 1 domain is approximately equally homologous to that of other cadherin EC 1 domains, and is not more closely related to mouse M-cadherin. However, it is considerably more homologous to cadherins than to DG I, thus explaining its distinct derivation from the latter. The affiliation of this molecule thus remains open to question. Further phylogenetic analysis using the sequence of other protein domains would help resolve it, as well as strengthen the topology of the tree presented here.

2.4 Conclusion

The data described above demonstrate that cadherins form a heterogeneous superfamily of molecules with unique regulatory and, possibly, distinct functional features. As with many other gene families, the superfamily appears to have been generated by successive rounds of gene duplication. While this mechanism usually resulted in the generation of a novel full-length cadherin gene, it may also have

resulted in incomplete duplications, yielding partially deleted cadherins such as T-cadherin. Such scenarios will now be easier to study following the description of several cadherin genes[23-26].

The sequence data allow the following generalizations. All bona fide cadherins follow the same tandem repeat structure in their extracellular portion. No cadherin has yet been isolated which harbours less than four tandemly-repeated cadherin domains, followed by a poorly-conserved premembrane domain. The vast majority of cadherins and cadherin-related molecules (e.g., desmoglein) isolated so far follow this pattern. The exception is the *Fat* gene product, a transmembrane protein isolated from *Drosophila melanogaster* with several features characteristic of cadherins[27]. However, because it is so different from other cadherins (it has 34 cadherin-like repeats), additional data such as the demonstration that this molecule can mediate calcium-dependent cell adhesion will be necessary to confirm its identity as a cadherin.

While most cadherins sequenced so far have retained a CAR site based upon the HAV motif, some have deviated substantially from this model. This is reflected in their independent phylogenies (e.g., M- and T-cadherin, Fig. II:2B). It is reasonable to postulate that this heterogeneity in the CAR site may confer different adhesive specificities than is observed in cadherins sharing the HAV-based CAR site. Other roles, apart from intercellular adhesion, may yet be ascribed to cadherins, including possibly some form of cell division control[27].

How old are cadherins? Can they be traced back to the first adhesion molecules which allowed the birth of metazoans? Examination of the protein structure

of cadherins suggests that they are built upon a tandemly repeated domain structure. If some form of exon shuffling has occurred, can the 'original' domain be found? So far, examination of cadherin gene structures indicates that a simple one-to-one assignment of protein domains to specific exons cannot be made[24,25], and that several recombinational steps may have been involved to produce the current protein domains. With one exception, the searching of sequence databases has not detected other proteins with sequence similarity to cadherins (Y. Pouliot, unpublished results). The sole exception is the discovery of a region of 32 amino acids in influenza strain A hemagglutinins with significant sequence similarity to the HAV region of cadherins[28]. The site includes the HAV motif and appears to be active since mutations in this region alter the functional properties of the hemagglutinin trimer [28]. A mechanism to explain the presence of the cadherin HAV site in such an unlikely candidate as influenza hemagglutinin remains to be proposed. Other proteins may yet be found to harbour regions of sequence similarity with the cadherins. With the rapid accumulation of sequence data from additional cadherin subtypes and the identification of molecular relatives of cadherins, it should become possible to assemble an extensive molecular evolutionary history of the cadherin superfamily and their molecular relatives.

2.5 Acknowledgements

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Fig. II:1. Structure of integral membrane and GPI-linked cadherins.

The primary structure of mature integral membrane (A) or GPI-linked (B) cadherins is depicted. See text for details. EC: extracellular domain; hashed region: transmembrane domain; CP: cytoplasmic domain.

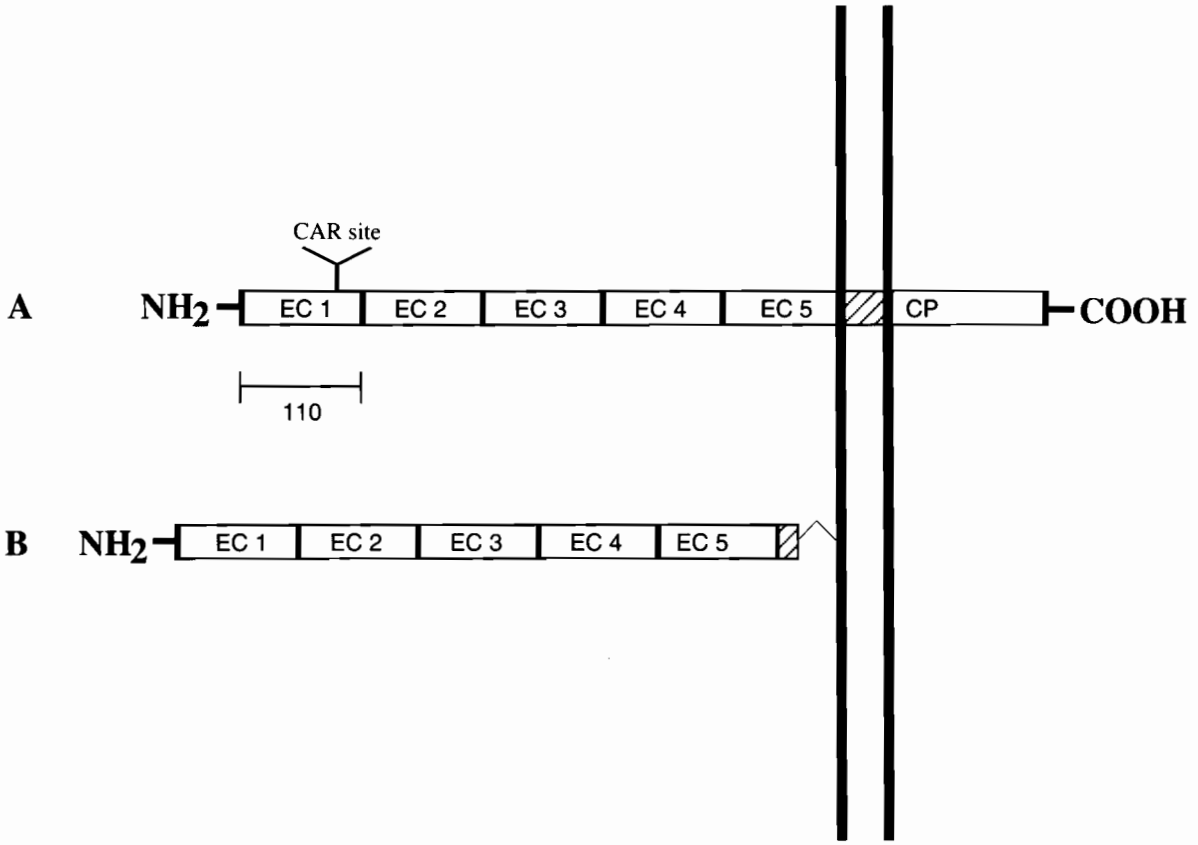
CAR site
A
NH2
EC 1
EC 2
EC 3
EC 4
EC 5
CP
COOH
110
B
NH2
EC 1
EC 2
EC 3
EC 4
EC 5

Fig. II:2. Phylogenetic analysis of the cadherins listed in Table II:1.

The primary structure of the EC 1 domain of the cadherins listed in Table II:1 was used to generate the phylograms in Figure II:1 according to the sequence alignment shown in Figure II:3. A set of 18 equally parsimonious trees was generated, of which a representative tree is shown in part A. Part B shows the consensus tree generated from this set according to a majority rule in which the branch assignments shown are followed in at least 2/3 of the trees. The trees are unrooted and were produced using the maximum parsimony method as implemented in the PAUP phylogenetic analysis program[36]. The heuristic search method was used, as the number of sequences is too large for an exact search method. Numbers along branches in part A. indicate the number of amino acid substitutions between a sequence and the branch point representing the deduced ancestral sequence. Numbers along branches in part B. indicate the percentage of trees whose branch assignments follow that of the tree shown. A limited description of human cadherins 4, 8, and 11 was originally provided in reference 7. The EC 1 sequence of these cadherins, and that of the newly-isolated cadherins 12 and 13, were graciously provided by S. Suzuki prior to publication (personal communication). Since an extensive characterization of these cadherins had not yet been published at the time of writing, they will not be addressed further. The EC 1 sequences of human[21] and bovine[37] desmoglein I and human desmocollin II/III[21] are included for comparison (the amino termini of desmocollin II and III are identical; alternative mRNA splicing generates different c-termini). CadN: N-cadherin; CadR: R-cadherin, CadP: P-cadherin; CadE: E-cadherin; CadB: B-cadherin; CadEP: EP-cadherin; CadT: T-cadherin; CadM: M-cadherin; L-CAM: Liver cell adhesion molecule; DGI: desmoglein I; DGII: desmosomal glycoprotein II/III (desmocollin II/III); Hs: *Homo sapiens;* Mm: *Mus musculus;* Gg: *Gallus gallus;* Bt: *Bos taurus;* X1: *Xenopus laevis.*

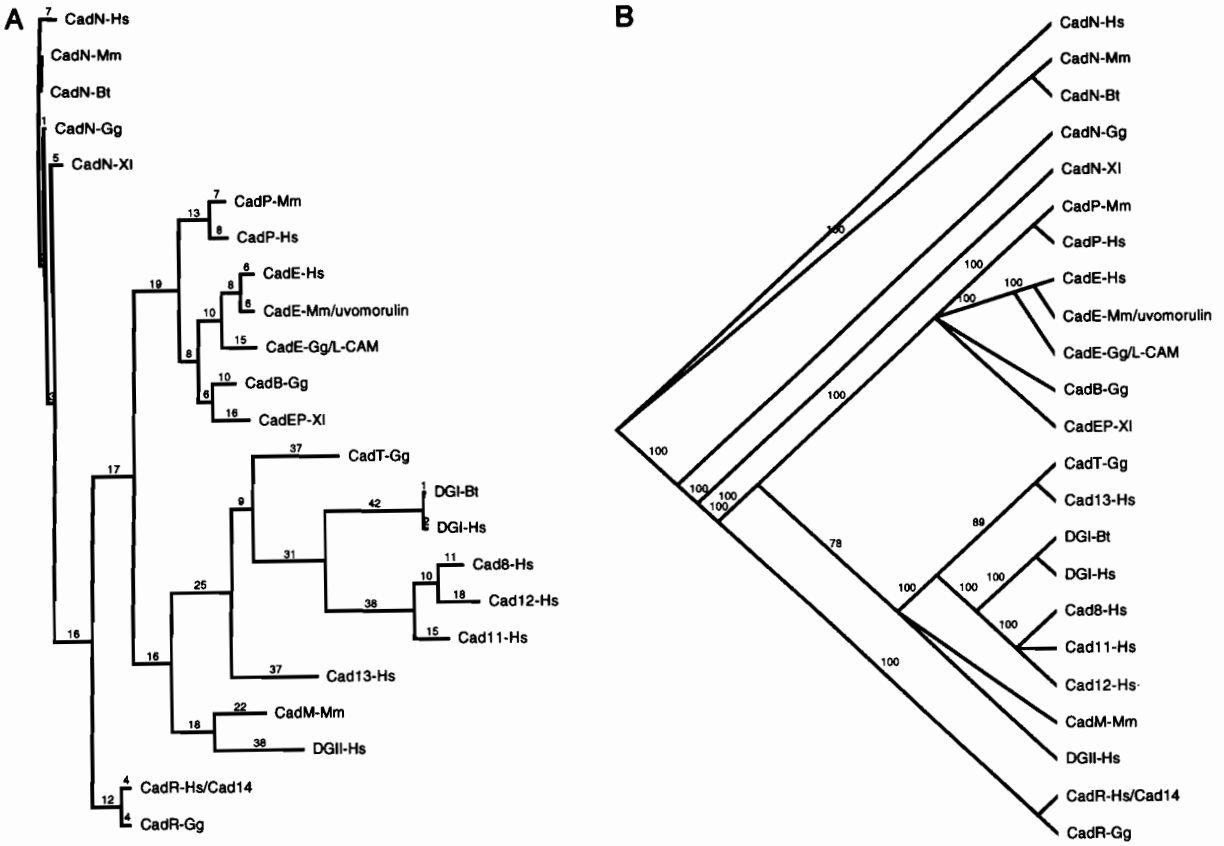
A
CadN-Hs
CadN-Mm
CadN-Bt
CadN-Gg
CadN-Xl
CadP-Mm
CadP-Hs
CadE-Hs
CadE-Mm/uvomorulin
CadE-Gg/L-CAM
CadB-Gg
CadEP-Xl
CadT-Gg
DGI-Bt
DGI-Hs
Cad8-Hs
Cad12-Hs
Cad11-Hs
Cad13-Hs
CadM-Mm
DGII-Hs
CadR-Hs/Cad14
CadR-Gg
B
CadN-Hs
CadN-Mm
CadN-Bt
CadN-Gg
CadN-Xl
CadP-Mm
CadP-Hs
CadE-Hs
CadE-Mm/uvomorulin
CadE-Gg/L-CAM
CadB-Gg
CadEP-Xl
CadT-Gg
Cad13-Hs
DGI-Bt
DGI-Hs
Cad8-Hs
Cad11-Hs
Cad12-Hs
CadM-Mm
DGII-Hs
CadR-Hs/Cad14
CadR-Gg

Fig. II:3. Sequence alignment of the EC 1 domain of cadherins listed in Table II:1.

The alignment of the EC 1 domain of cadherins listed in Table II:1 and used to generate the phylograms depicted in Figure II:1 is shown. The alignment was generated manually and is centred around well-conserved sites such as the LDRE and HAV motifs.

```
CadN-Hs         DWVIPPINLPENSRG-PFPQELVRIRSDRDKNLSLRIRVTGPGADQPPTGIFILNPISGQLSVTKPLDRQQNARFHLGAHAVDINGNQVETP--IDIVINVIDMNDNRPEF
CadN-Mm         DWVIPPINLPENSRG-PFPQELVRIRSDRDKNLSLRYSVTGPGADQPPTGIFIINPISGQLSVTKPLDRELIARFHLRAHAVDINGNQVENP--IDIVINVIDMNDNRPEF
CadN-Bt         DWVIPPINLPENSRG-PFPQELVRIRSDRDKNLSLRYSVTGPGADQPPTGIFIINPISGQLSVTKPLDRELIARFHLRAHAVDINGNQVENP--IDIVINVIDMNDNRPEF
CadN-Gg         DWVIPPINLPENSRG-PFPQELVRIRSDRDKSLSLRYSVTGPGADQPPTGIFIINPISGQLSVTKPLDREQIASFHLRAHAVDVNGNQVENP--IDIVINVIDMNDNRPEF
CadN-Xl         DWVIPPINVPENARG-TFPQELVRIRSDRDKNLSLRYSVTGPGADQPPIGVFIINPIGGQLSVTKPLDREQIANFHLRAHAVDVNGNQVENP--IDIVINVIDMNDNRPEF
CadP-Mm         EWVMPPIFVPENGKG-PFPQRLNQLKSNKDRGTKIFYSITGPGADSPPEGVFTIEKESGWLLLHMPLDREKIVKYELYGHAVSENGASVEEP--MNISIIVTDQNDNKPKF
CadP-Hs         DWVVAPISVPENGKG-PFPQRLNQLKSNKDRDTKIFYSITGPGADSPPEGVFAVEKETGWLLLNKPLDREEIAKYELFGHAVSENGASVEDP--MNISIIVTDQNDHKPKF
CadE-Hs         DWVIPPISCPENEKG-PFPKNLVQIKSNKDKEGKVFYSITGQGADTPPVGVFIIERETGWLKVTEPLDRERIATYTLFSHAVSSNGNAVEDP--MEILITVTDQNDNKPEF
CadE-Mm/uvomo   DWVIPPISCPENEKG-EFPKNLVQIKSNRDKETKVFYSITGQGADKPPVGVFIIERETGWLKVTQPLDREAIAKYILYSHAVSSNGEAVEDP--MEIVITVTDQNDNRPEF
CadE-Gg/L-CAM   DWVIPPISCLENHRG-PYPMRLVQIKSNKDKESKVYYSITGQGADSPPVGIFIIERETGWLEVTEQLDREKIDRYTLLSHAVSASGQPVEDP--MEIIITVMDQNDNKPVF
CadT-Gg         AILATPILIPENQRP-PFP-RSVGKVIRSEGTEGAKFRLSGKGVDQDPKGIFRINEISGDVSVTRPLDREAIANYELEVTDLSGK--IIDGPVRLDISV--IDQNDNRPMF
CadM-Mm         AWVIPPISVSENHKRLPYP--LVQIKSDKQQLGSVIYSIQGPGVDEEPRNVFSIDKFTGRVYLNATLDREKTDRFRLRAFALDLGGSTLEDP--TDLEIVVVDQNDNRPAF
CadR-Hs/Cad4    DWVIPPINVPENSRG-PFPQQLVRIRSDKDNDIPIRYSITGVGADQPPMEVFSINSNSGRMYVTRPMDREEHASYHLRAHAVDMNGNKVENP--IDLYIYVIDMNDNMPEF
CadR-Gg         DWVIPPINVPENSRG-PFPQQLVRIRSDKDKEIHIRYSITGVGADQPPMEVFSIDPVSGRMYVTRPMDREERASYHLRAHAVDMNGNKVENP--IDLYIYVIDMNDNRPEF
CadB-Gg         DWVIPPIKVPENERG-PFPKNLVQIKSNRDREAKIFYSITGQGADAPPEGIFTIEKETGWMKVTQPLDREHINKYHLYSHAVSENGKPVEEP--MEIIVTVTDQNDNKPQF
CadEP-Xl        DWVIPPIKVSENERG-PFPKRLVQIKSNKDRFNKVYYSITGQGADNPPQGVFRIEWETGWMLVTRPLDREEYDKYVLSSHAVSENGSPVEEP--MEITINVIDQNDNRPKF
DGI-Bt          EWIKFAAACREGEDN-SKRNPIAKIHSDCAANQQVTYRISGVGIDQPPYGIFVINQKTGEINITSIVDREVTPFFVIYCRALNSLGQDLEKP--LELRVRVLDINDNPPVF
DGI-Hs          EWIKFAAACREGEDN-SKRNPIAKIHSDCAANQQVTYRISGVGIDQPPYGIFVINQKTGEINITSIVDREVTPFFIIYCRALNSMGQDLERP--LELRVRVLDINDNPPVF
DGII-Hs         RWAPIPCSMLENSLG-PFPLFLQQVQSDTAQNYTIYYSIRGPGVDQEPRNLFYVERDTGNLYCTRPVDREQYESFEIIAFATTPDGYTPELP-LPLIIIKIEDENDNYPIF
Cad8-Hs         GWVWNQKFVLEEFSG-PEPILVGRLHTDLDPGSKKIKYILSGDGAGTIFQINDVTGDIHAIK---RLDREEKARYTLTAQAVDWETSKPLEP-PSEFIIKVQDINDNAPEF
Cad11-Hs        GWVWNQFFVIEEYTG-PDPVLVGRLHSDIDSGDGNIKYILSGEGAGTIPVIDDKSGNIHATK---TLDREERAQYTLMAQAVDRDTNRPLEP-PSEFIVKVQDINDNPPEF
Cad12-Hs        GWVWNQFFVLEEYVG-SEPQYVGKLHSDLDKGEGTVKYTLSGDGAGTVFTIDETTGDIHAIR---SLDREEKPFYTLRAQAVDIETRKPLEP-ESEFIIKVQDINDNEPKF
Cad13-Hs        SIVVSPILIPENQRQ-PFPRDVGKVVDSDRPERSKFRLTGKGVDQEPKGGIFRINENTGSVSVTRTLDREVIAVYQLFVETTDVNGKTLEGP-VPLEVIVI-DQNDNRPIF
```

Fig. 3. Sequence alignment of the EC 1 domain of cadherins listed in Table 1. The alignment of the EC 1 domain of cadherins listed in Table 1 and used to generate the phylograms depicted in Figure 1 is shown. The alignment was generated manually and is centred around well-conserved sites such as the LDRE and HAV motifs.

3.0 IDENTIFICATION OF A CADHERIN CELL ADHESION RECOGNITION SEQUENCE[2]

FOREWORD

The following Chapter describes results from a collaborative effort with Drs. Orest Blaschuk (Royal Victoria Hospital), Sam David (Montreal General Hospital) and Riaz Farookhi (Department of Physiology, McGill University) aimed at identifying the cadherin CAR site. The strategy described here relies upon the perturbation of cellular processes known to be cadherin-mediated, such as embryo compaction and neurite extension (Vestweber and Kemler, 1984; Doherty et al., 1991a, respectively) by synthetic peptides encoding regions conserved in the cadherin family. Dr. Blaschuk hypothesized that synthetic peptides with a sequence derived from a site involved in mediating these interactions might retain sufficient tertiary conformation to interfere with cadherin-mediated intercellular adhesion. Shortly after this paper was published, Nose et al. (1990) showed that mutating a single amino acid residues immediately flanking the HAV tripeptide on either side resulted in the loss of cadherin subtype specificity as measured by in vitro assays. Synthetic peptides encoding the HAV site have since been shown to block avian myoblast differentiation (Mege et al., 1992), in addition to neurite extension (Doherty et al., 1991a). Furthermore, Doherty et al. (1991a) used an additional control in the form of a peptide with the same residue composition as the HAV peptide, but whose sequence has been scrambled (nonsense

Sullivan, R

[2] Published as: Blaschuk, O.W., Sullivan, R., David, S. & Pouliot, Y. Dev. Biol. **139**:227-229 (1990)

peptide). As expected, this peptide lacks any perturbing activity. These results demonstrate that HAV-containing peptides can be used as reagents with which to perturb cadherin-mediated processes.

3.1 Summary

The molecular mechanisms by which the cadherins interact with one another to promote cell adhesion have not been elucidated. In particular, the amino acid sequences of the cadherin cell adhesion recognition sites have not been determined. Here we demonstrate that synthetic peptides containing the sequence HAV, which is common to all of the cadherins, inhibit two processes (compaction of eight-cell-stage mouse embryos and rat neurite outgrowth on astrocytes) that are known to be mediated by cadherins. The data suggest that the tripeptide HAV is a component of a cadherin cell adhesion recognition sequence.

3.2 Introduction

The cadherins are a family of integral membrane glycoproteins that mediate calcium-dependent, vertebrate cell adhesion (Takeichi, 1988). These cell adhesion molecules (CAMs) are believed to promote cell adhesion through homophilic interactions (Hatta et al., 1988; Nose et al., 1988), although several studies have shown that the cadherins are also capable of forming heterotypic complexes with one another under certain circumstances (Volk et al., 1987; Miyatani et al., 1989). These latter observations suggest that the cadherins contain a common cell adhesion recognition

(CAR) sequence. Such a sequence may reside within the first extracellular domain (designated EC1) of each of the cadherins, as monoclonal antibodies directed against this domain have been shown to inhibit cadherin-mediated cell adhesion (Hatta et al., 1988; Miyatani et al., 1989). The CAR sequences of several cell and substrate adhesion molecules are known (Martin and Timpl, 1987; Ruoslahti and Pierschbacher, 1987). In general, CAR sequences are composed of at least three amino acid residues. The most rigorously investigated CAR sequence is RGD. This sequence is found in fibronectin and many other adhesion molecules. The hypothesis which emerges from these observations suggests that a CAR sequence common to all of the cadherins should be present within their EC1 domains and that this sequence should be composed of at least three amino acid residues.

Our examination of the EC1 domains of four well-characterized cadherins (liver-CAM, E-, P-, and N-cadherin) revealed the presence of three potential cadherin CAR sequences with the aforementioned properties: PPI, GAD, and HAV (Blaschuk et al., 1990; Hatta et al., 1988). We have previously shown that the HAV-containing regions of the cadherin EC1 domains are homologous to the amino-termini of the HA1 chains of influenza strain A hemagglutinins (Blaschuk et al., 1990). Each of these homologous regions extends over 40 amino acids. A subgroup of these hemagglutinins contain amino-terminal regions that harbour the tripeptide sequence, HAV. These regions stabilize the interaction between the HA1 and HA2 chains of the hemagglutinins (Daniels et al., 1985; Wiley and Skehel, 1987). Mutations in these regions result in an altered conformation of the hemagglutinin and a lower infectivity

of the virus.

The extensive homology between the cadherins and hemagglutinins in the HAV-containing regions suggests that this region may play an important role in modulating the function of the cadherins. In this report we demonstrate that synthetic peptides containing the sequence HAV, which is found in the EC1 domains of all cadherins, inhibit the compaction of mouse embryos and neurite outgrowth on astrocytes. Both of these processes are known to be mediated by cadherins (Shirayoshi et al., 1983; Neugebauer et al., 1988, Tomaselli et al., 1988). The results suggest that the tripeptide HAV is a component of a cadherin CAR sequence.

3.3 Methods

3.31 Mouse embryo compaction assays.

The techniques utilized to obtain and culture the mouse embryos are described in detail by Hogan et al. (1986). Female CD-1 mice (Charles River, Kingston, NY) were mated with CD-1 males. The morning of the vaginal plug was considered Day 0 of gestation. The females were sacrificed on Day 2 of gestation and the oviducts were flushed with Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories, Grand Island, NY) supplemented with sodium bicarbonate (3.7 mg/ml), 10 m*M* HEPES, and 4% bovine serum albumin. The 8- to 16-cell-stage embryos were recovered and washed in DMEM. The embryos were then cultured under oil in 50 µl drops of DMEM containing synthetic peptides at a final concentration of 1 mg/ml. The synthetic peptides (two decapeptides and two hexamers) were purchased from

Multiple Peptide Systems (San Diego, CA). The amino acid sequences of the two decapeptides (LRAHAVDVNG-amide and VIPPINLPEN-amide) were derived from the first extracellular domain (EC1) of avian N-cadherin (amino acid residues 240-249 and 167-176, respectively) (Hatta et al., 1988). Each decapeptide contains a tripeptide (HAV and PPI, respectively) common to all of the cadherins analyzed to date. The amino acid sequence of one of the hexamers (AHAVSE-amide) is a composite of several cadherin amino acid sequences. It contains the tripeptide HAV, which is common to all cadherins, as well as three nonconserved residues. The amino acid sequence of the other hexamer (IPPINL-amide) is identical to that found in avian N-cadherin. The embryos were examined after 24 hr of culture at 37°C in 5% CO_2. Two independent experiments (each utilizing 10-14 embryos) were conducted with each peptide.

3.32 Neurite outgrowth assays

Newborn rat dorsal root ganglia were incubated for 30 min in 0.125% trypsin and collagenase in calcium- and magnesium-free Hanks' balanced salt solution. Trypsin inhibitor (50 μg/ml) and DNase (40 μg/ml) were then added and the dissociated cells were preplated for 2 hr in a tissue culture dish precoated with fetal bovine serum. The neurons were plated in chemically defined serum-free medium (Bottenstein and Sato, 1978) containing nerve growth factor (25 ng/ml) and synthetic peptides (1 mg/ml) onto astrocyte monolayers growing on 12-mm round coverslips (10,000 cells were plated per coverslip). The astrocytes were prepared using protocols

described by David (1988). The neuron-astrocyte cultures were fixed after 18 hr with 4% paraformaldehyde for 15 min at room temperature and then with 95% ethanol/5% acetic acid for 20 min at -20°C. The neurons in these cultures were identified by immunofluorescence microscopy using a monoclonal anti-neurofilament antibody as described by Wood and Anderton (1981). The percentage of large neurons extending neurites greater than two cell bodies in length was determined by phase-contrast and immunofluorescence microscopy. Approximately 100 neurons were examined on each coverslip. Two coverslips were viewed in each of three separate experiments. A total of six coverslips were examined for each peptide that was tested.

3.4 Results and discussion

The strong homologies between the HAV-containing regions of the cadherins and functional regions of the hemagglutinins prompted us to investigate the effects of HAV-containing synthetic peptides on cadherin-mediated cell adhesion. We initially examined the ability of two synthetic decapeptides (LRAHAVDVNG and VIPPINLPEN) to inhibit the compaction of eight-cell-stage mouse embryos and rat neurite outgrowth on astrocytes. The amino acid sequences of these two decapeptides were derived from the EC1 domain of avian N-cadherin (amino acid residues 240-249 and 167-176, respectively) (Hatta et al., 1988). Each decapeptide contains a tripeptide (HAV and PPI, respectively) common to all of the cadherins analyzed to date. The compaction of mouse embryos has been shown to be mediated by E-cadherin (Shirayoshi et al., 1983), whereas N-cadherin is known to be involved in mediating

neurite outgrowth on astrocytes (Negebauer et al., 1988, Tomaselli et al., 1988; David and Blaschuk, unpublished results).

The decapeptide, VIPPINLPEN, had no effect upon either the compaction of eight-cell-stage embryos or neurite outgrowth on astrocytes (Tables III:1 and III:2). In contrast, the decapeptide LRAHAVDVNG completely prevented the compaction of embryos. It also inhibited neurite outgrowth by approximately 40% relative to the controls. These results are consistent with previous observations regarding the inhibitory effects of anti-cadherin antibodies on compaction and neurite outgrowth. Complete inhibition of embryo compaction can be achieved with monoclonal antibodies directed against E-cadherin (Shirayoshi et al., 1983), whereas anti-N-cadherin antibodies do not totally prevent neurite outgrowth on astrocytes (Neugebauer et al., 1988; Tomaselli et al., 1988; David and Blaschuk, unpublished results).

To determine if the peptides were toxic to the cells, we investigated the effects of the peptides on cell division. Mouse embryos at the two-cell-stage of development were cultured in the presence of each of the peptides (1 mg/ml) for 48 hr. Cell division was not inhibited by any of the peptides.

The ability of the HAV-containing decapeptide to inhibit cadherin-mediated cell adhesion indicates that it harbours a CAR sequence. To further delineate this sequence, we examined the ability of a hexapeptide, AHAVSE, to inhibit compaction of embryos and neurite outgrowth. The amino acid sequence of this hexapeptide is a composite of several cadherin amino acid sequences (Hatta et al., 1988). It contains the tripeptide HAV, which is common to all cadherins, as well as three nonconserved

amino acid residues. The hexapeptide inhibited compaction in 70% of the embryos and reduced neurite outgrowth by approximately 38% relative to the controls (Tables III:1 and III:2). Although the hexapeptide was an effective inhibitor of cadherin-mediated cell adhesion, it was a less potent inhibitor than the HAV-containing decapeptide. These results suggest that the amino acid sequence HAV is an essential component of a cadherin CAR sequence. Furthermore, the data indicate that the nonconserved residues bordering the HAV sequence may also be involved in modulating cadherin interactions. M. Takeichi has recently found that alterations in the nonconserved amino acid residues immediately adjacent to the tripeptide HAV do not abolish cadherin-mediated cell adhesion, but affect the binding specificities of the cadherins (personal communication). These observations support the notion that the CAR sequence of each cadherin subtype extends beyond the tripeptide HAV.

The identification of a CAR sequence component common to all of the cadherins provides an explanation for the ability of the cadherins to form heterotypic complexes (Volk et al., 1987; Miyatani et al., 1989). Synthetic peptides containing the tripeptide HAV should serve as useful probes for investigating the role of the cadherins in embryogenesis and other developmental processes. The existence of homologies between the HAV-containing regions of the cadherins and functional domains of the hemagglutinins leads us to speculate that similar cell adhesion domains may be present within other CAMs.

3.5 References

References for this paper can be found in the reference section (Chapter 8).

Table III:1: Effect of synthetic peptides on the compaction of mouse embryos

Peptide	Number of embryos examined	Number of embryos compacted after 24 hr	Percentage of embryos compacted
None	22	20	91
LRAHAVDVNG	23	0	0
VIPPINLPEN	24	23	96
AHAVSE	23	7	30
IPPINL	22	21	95

Table III:2: Effect of synthetic peptides on neurite outgrowth on astrocytes

Peptide	Percentage of neurons extending neurites[a]
None	61 ± 3
LRAHAVDVNG	37 ± 4
VIPPINLPEN	68 ± 5
AHAVSE	38 ± 5
IPPINL	64 ± 7

[a]Mean of three separate experiments and the standard error.

4.0 - IDENTIFICATION OF A CONSERVED REGION COMMON TO CADHERINS AND INFLUENZA STRAIN A HEMAGGLUTININS[3]

FOREWORD

While attempting to identify the cadherin CAR site, I hypothesized that other molecules might harbour a similar site if indeed it is functionally important. However, for several years after the first cadherins were cloned, no sequence similarities with the cadherins could be identified in the protein sequence databases. Nonetheless, using several database search techniques I searched the sequence databases for proteins with sequence similarity to the cadherin CAR site. In this way, I discovered that influenza strain A hemagglutinins harbour a region of similarity to the cadherin CAR site. This report, presented in this Chapter, was the first to show a sequence similarity between cadherins and another family of proteins. Particularly important was the observation that the region of similarity in hemagglutinin is also functional. Thus, the sequence encoding the cadherin CAR site is active in both families of proteins.

4.1 Summary

Cadherins are a family of integral membrane glycoproteins that mediate homophilic, calcium-dependent cell adhesion in vertebrate species. The primary structures of six members of the cadherin family have recently been determined. The extracellular portion of these proteins is composed of five domains, the first of which

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is the most highly conserved among cadherins. Previous searches of protein sequence databases have revealed little or no sequence homology between cadherins and other proteins. Here we report that the first extracellular domain of cadherins exhibits substantial sequence homology with the amino termini of influenza strain A hemagglutinins. These regions of sequence homology have been shown to be functionally important in both cadherins and hemagglutinins. Our observations suggest that a functional domain of cadherins is conserved among other proteins.

4.2 Results and discussion

The first extracellular domain of cadherins contains a region with a consensus sequence characterized by a conserved tripeptide, HAV (Fig. IV:1; Hatta *et al.*, 1988). We shall refer to this region as the HAV region. The substitution of other residues for amino acids immediately flanking the tripeptide, HAV, in mouse N-cadherin abolishes homotypic binding (M. Takeichi, personal communication). Furthermore, synthetic peptides containing the HAV sequence inhibit cadherin-mediated adhesion processes such as embryo compaction and neurite extension (Blaschuk *et al.*, unpublished results). Lastly, monoclonal antibodies that inhibit cadherin-mediated cell adhesion appear to be exclusively directed against epitopes localized to the first extracellular domain (Hatta *et al.*, 1988). These results demonstrate that the HAV region constitutes a cadherin cell adhesion site.

The consensus sequence for the HAV region of cadherins was used as a search key to scan the National Biomedical Research Foundation (NBRF) protein sequence

database. This search revealed that the amino-terminal domains of strain A hemagglutinins could be aligned remarkably well with the HAV regions of cadherins.

Influenza strain A hemagglutinins are integral membrane proteins that mediate attachment and subsequent fusion of the virus with the epithelia of the upper respiratory tract of a large variety of mammals and birds (for a review, see Wiley & Skehel, 1987). The mature protein is a homotrimeric complex. Each subunit of the complex is composed of two covalently linked chains (designated HA1 and HA2). The region of the HA1 chain beginning at residue positions 12 and 1 in Figures IV:1 and IV:2 respectively, is common to all HA1 chains illustrated. It is characterized by the presence of a conserved dipeptide, HA, followed by either Val or Asn (Figs. IV:1 and IV:2). We will refer to this region as the HA(V/N) region. In the case of group 1 hemagglutinins, the sequence homology extends over 32 residues starting from position 12 (Fig. IV:1). Several residues conserved among cadherins were also found to be conserved among hemagglutinins, while other residues conserved among cadherins have been substituted for amino acids considered homologous by the Dayhoff Mutation Data Matrix (i.e. with positive scores) (Dayhoff *et al.*, 1983). Furthermore, partially conserved amino acids are also abundant. Overall, the HA(V/N) domain of group 1 hemagglutinins includes 25% of residues that are identical to the consensus sequence for the cadherin family, with an additional 25% of residues being either partially conserved or homologous according to the Dayhoff matrix (Fig. IV:1). For group 2 hemagglutinins the figures are 13% and 30%, respectively (Fig. IV:2). An example of an alignment between an individual cadherin and hemagglutinin is presented in Figure

IV:3. In this case, 30% of residues are identical, 15% are conservative substitutions, and the significance of the alignment score is 3.55 standard deviations.

As with the HAV region of cadherins, the HA(V/N) region of hemagglutinins is known to be functionally important. Crystallographic studies of strain X-31 hemagglutinin indicate that the His residue at position 18 (Fig. IV:1) of the HA1 chain forms hydrogen bonds with two Ile residues in the amino terminus of the HA2 chain (Daniels *et al.*, 1985). This results in the amino terminus of the HA2 chain being buried within the hemagglutinin homotrimeric complex (Daniels *et al.*, 1985). Fusion of the influenza strain A virus with the host cell relies upon a pH-dependent conformational change of hemagglutinin (Wiley & Skehel, 1987). The pH at which this change occurs is raised by mutations that substitute other residues for His18, resulting in lower infectivity for the mutant virus (this residue is referred to as His17 by Daniels *et al.*, 1985).

Both hemagglutinins and cadherins exhibit considerable intra-family protein sequence heterogeneity. Results described by Webster *et al.* (1983) indicate that the average homology for the first 16 residues of the HA1 chains illustrated in Figures IV:1 and IV:2 is only 43%. For cadherins the mean overall protein sequence homology is approximately 50% (Hatta *et al.*, 1988). Hemagglutinins are rapidly evolving proteins. The discovery of a region of sequence homology between the rapidly evolving hemagglutinins and the diverse family of cadherins is therefore noteworthy.

The high degree of conservation between the HA(V/N) regions of

hemagglutinins and cadherins constitutes further evidence that these regions play an important role in modulating the function of these two families of membrane proteins. We speculate that additional cell adhesion molecules will be discovered to contain domains similar to these two regions.

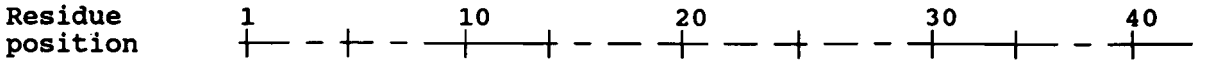
We thank Drs. C. Richardson, K. Hastings and N. Cashman for helpful discussions.

4.3 References

References for this paper can be found in the reference section (Chapter 8).

Fig. IV:1. Local alignment of cadherins with group 1 hemagglutinins of influenza strain A.

Periods indicate gaps inserted to obtain optimal alignment either between hemagglunins or between hemagglutinins and cadherins. Boxes indicate residues that are perfectly or near perfectly conserved between hemagglutinins and cadherins. Letters printed in bold and asterisks in the consensus sequence indicate partial conservation or conservative substitutions, which score a value of 1 or greater in the Dayhoff matrix. Lower case letters in consensus sequences indicate the predominant residue (> 75% of cases) when a position is partially conserved. the number immediately following the name of the protein or file name refers to the position of the 1st residue in each sequence presented, when known. The top graduated scale provides an arbitrary numbering scheme for reference in the text. Hemagglutinin sequences were obtained from the NBRF protein database (release 18) and are identified by their NBRF file name. Note that all hemagglutinin sequences presented start with the amino terminus of the mature protein. We found that based on the sequence of their N-termini, they could be assembled into two groups denoted group 1 and group 2 hemagglutinins. Cadherin sequences are reproduced from Hatta *et al.* (1988), Miyatani *et al.* (1989), and Mansouri *et al.* (1988). Cadherins and hemagglutinins were aligned manually and with the GENALIGN computer program (Intelligenetics, Calif.) using the Needleman-Wunsch algorithm. Hemagglutinins were originally identified using the ProfileSearch program (University of Wisconsin Genetics Computer Group (UWGCG), version 5.3, Devereux *et al.*, 1984) with the HAV region of L-CAM, mE-cadh, cN-cadh and mP-cadh as a search key. The UWGCG package was implemented by the Canadian Institute for Scientific and Technical Information (CISTI). Sequences illustrated: HMIV6: A/England/321/77; A/Bangkok/1/79. HMIV10: A/Duck/Manitoba/53 [H10]. HMIVV: A/Victoria/3/75; A/England/321/77. HMIVH: A/NT/60/68/29C; A/Aichi/2/68; X-31 [H3]; A/Memphis/102/72. HMIVH7: A/Turkey/Oregon/71 [H7]. HMIVDU: A/Duck/Ukraine/63. HMIVF: fowl influenza. LCAM: liver cell adhesion molecule (chicken E-cadherin); cN-cadh: chicken N-cadherin; mN-cadh: mouse N-cadherin; mE-cadh: mouse E-cadherin; hE-cadh: human E-cadherin; mP-cadh: mouse P-cadherin. HA: hemagglutinin.



Group 1 hemagglutinins

HMIVF;19		P	GN	D	DKIC	L	GH	HAV	S.	NG	TK	V	NTLTERGVE	V	VNATE
HMIVH;17	QDL	P	GN	D	.NNTATLC	L	GH	HAV	P.	NG	TL	V	KTITDDQIE	V	TNATE
HMIV6;17	QNL	P	GN	D	.NSTATLC	L	AH	HAV	P.	NG	TL	V	KTITNDQIE	V	TNATE
HMIVV;17	QDL	P	GN	D	NNSTATLC	L	GH	HAV	P.	NG	TL	V	KTITNDQIE	V	TNATE
HMIVDU;17	QDL	P	GN	D	.NSTATLC	L	GH	HAV	P.	NG	TI	V	KTITDDQIE	V	TNATE
HMIVH3;22	QYL	P	GN	D	.NSTATLC	L	GH	HAV	P.	NG	TL	V	KTITNDQIE	V	TNATE
HMIVH7;19					DKIC	L	GH	HAV	A.	NG	TK	V	NTLTERGIE	V	VNATE
HMIV10;18					DKIC	L	GH	HAV	P.	NG	II	V	KTLTNEKEE	V	TNATE
Cadherins															
cN-cadh;229		P	.L	D	REQIASFH	L	RA	HAV	DV	NG	NQ	V	ENPIDIVIN	V	IDMND
mN-cadh;224		P	.L	D	RELIARFH	L	RA	HAV	DI	NG	NQ	V	ENPIDIVIN	V	IDMND
LCAM;168		Q	.L	D	REKIDRYT	L	LS	HAV	SA	SG	QP	V	EDPMEIIIT	V	MDQND
mE-cadh;221		P	.L	D	REAIAKYI	L	YS	HAV	SS	NG	EA	V	EDPMEIVII	V	TDQND
hE-cadh		P	.L	D	RERIATYT	L	FS	HAV	SS	NG	NA	V	EDPMEILIT	V	TDQND
mP-cadh;164		P	.L	D	REKIVKYE	L	YG	HAV	SE	NG	AS	V	EEPMNISII	V	TDQND
consensus for group 1 HA	Q L	p	GN	D	N TatlC	L	gH	HAV	p	NG	t	V	kTiT dqiE	V	tNATE
consensus for cadherins		p	L	D	RE Ia y	L		HAV	s	NG		V	E Pm I I	V	DqND
consensus for group 1 HA and cadherins		p		D	* *	L		HAV	*	NG		V	* *	V	** *

Fig. IV:2. Local alignment of cadherins with group 2 hemagglutinins of influenza strain A.

Group 2 hemagglutinins were aligned with cadherins as in Fig. 1. Sequences illustrated: HMIV: A/PR/8/34. HMIV2: A/Japan/305/57 [H2]. HMIV5: A/WSN/33 [HON1]. HMIVH2: A/RI/5-/57 [H2]. HMIVH3: A/Memphis/1/71 [H3]. HMIVH5: A/shearwater/Australia/75 [H5]. HMIVH6: A/shearwater/Australia/72 [H6]. HMIVN1: A/swine/NJ/11/76 [H1N1]. HMIVUR: A/USSR/90/77. LCAM: liver cell adhesion molecule (chicken E-cadherin;); cN-cadh: chicken N-cadherin; mN-cadh: mouse N-cadherin; mE-cadh: mouse E-cadherin; hE-cadh: human E-cadherin; mp-cadh: mouse P-cadherin. HA: hemagglutinin.

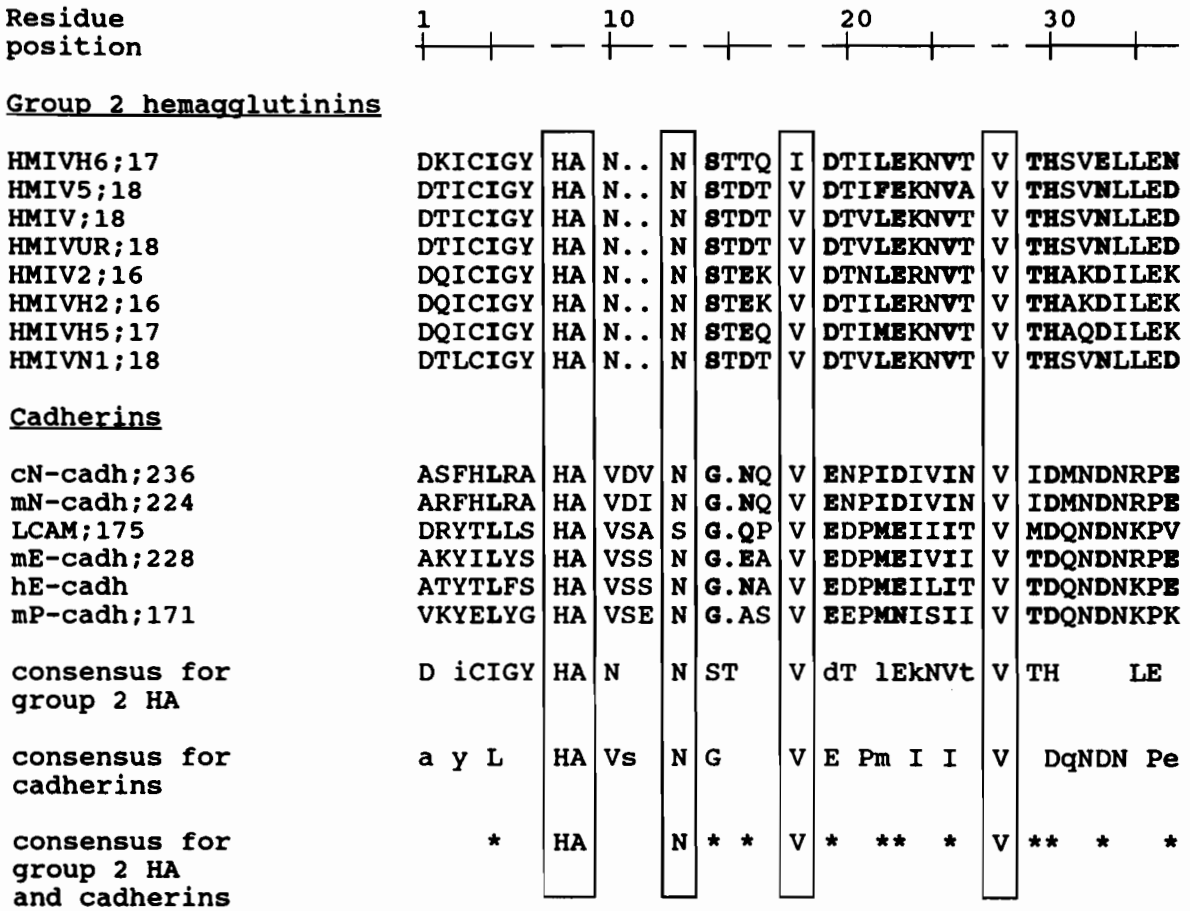
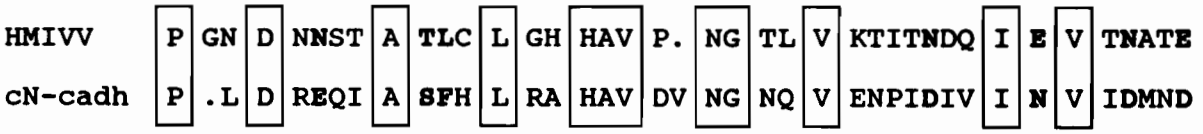

Residue position
1
10
20
30
Group 2 hemagglutinins
HMIVH6;17 DKICIGY HA N.. N STTQ I DTILEKNVT V THSVELLEN
HMIV5;18 DTICIGY HA N.. N STDT V DTIFEKNVA V THSVNLLED
HMIV;18 DTICIGY HA N.. N STDT V DTVLEKNVT V THSVNLLED
HMIVUR;18 DTICIGY HA N.. N STDT V DTVLEKNVT V THSVNLLED
HMIV2;16 DQICIGY HA N.. N STEK V DTNLERNVT V THAKDILEK
HMIVH2;16 DQICIGY HA N.. N STEK V DTILERNVT V THAKDILEK
HMIVH5;17 DQICIGY HA N.. N STEQ V DTIMEKNVT V THAQDILEK
HMIVN1;18 DTLCIGY HA N.. N STDT V DTVLEKNVT V THSVNLLED
Cadherins
cN-cadh;236 ASFHLRA HA VDV N G.NQ V ENPIDIVIN V IDMNDNRPE
mN-cadh;224 ARFHLRA HA VDI N G.NQ V ENPIDIVIN V IDMNDNRPE
LCAM;175 DRYTLLS HA VSA S G.QP V EDPMEIIIT V MDQNDNKPV
mE-cadh;228 AKYILYS HA VSS N G.EA V EDPMEIVII V TDQNDNRPE
hE-cadh ATYTLFS HA VSS N G.NA V EDPMEILIT V TDQNDNKPE
mP-cadh;171 VKYELYG HA VSE N G.AS V EEPMNISII V TDQNDNKPK
consensus for group 2 HA D iCIGY HA N N ST V dT lEkNVt V TH LE
consensus for cadherins a y L HA Vs N G V E Pm I I V DqNDN Pe
consensus for group 2 HA and cadherins * HA N * * V * ** * V ** * *

Fig. IV:3. Sequence alignment between HMIVV hemagglutinin and chicken N-cadherin.

The optimal alignment between the HAV regions of HMIVV hemagglutinin and chicken N-cadherin was generated using the interactive ALIGN program (Protein Identification Resource, NBRF) using the Dayhoff matrix with a gap penalty of 6 and a bias of 6. The significance of this alignment was 3.55 standard deviations.


HMIVV P GN D NNST A TLC L GH HAV P. NG TL V KTITNDQ I E V TNATE
cN-cadh P .L D REQI A SFH L RA HAV DV NG NQ V ENPIDIV I N V IDMND

5.0 DEVELOPMENTAL REGULATION OF A CADHERIN DURING THE DIFFERENTIATION OF SKELETAL MYOBLASTS[4]

FOREWORD

My M.Sc. thesis demonstrated that L_6 myoblasts exhibit calcium-dependent intercellular adhesion. At that time I had postulated that this activity was probably mediated by a cadherin (Pouliot, 1988). To begin investigating this possibility, we relied upon an immunological approach based upon a pan-cadherin antiserum produced by Dr. Orest Blaschuk. Immunological reagents available at the time were mostly species-specific monoclonal antibodies against individual cadherin subtypes. The antiserum used in the experiments described in this Chapter was raised against four synthetic peptides corresponding to highly conserved regions of cadherins (three from extracellular domains, one from the cytoplasmic region; see Blaschuk and Farookhi, 1989). CADH-1 was used to show that an immunoreactive protein was detectable in L_6 myoblasts and that this protein was regulated during their differentiation in vitro. I subsequently developed (with the help of Dr. Blaschuk) two additional polyclonal antisera, one of which was directed solely against a peptide encoding the CAR site of cadherin. Identical results were obtained with these other antisera (not shown).

[4] Published as: Pouliot, Y., Holland, P.C. & Blaschuk, P.C. Dev. Biol., 141:292-298 (1990)

5.1 Summary

Cadherins are a family of integral membrane glycoproteins which mediate calcium-dependent intercellular adhesion in vertebrate species. Here we present evidence that fusion-competent rat L_6 myoblasts express a cadherin (M_r 127 kDa). The levels of this cadherin were found to be developmentally regulated. Maximal levels were expressed prior to fusion. The increase in cadherin levels observed during differentiation was prevented by the differentiation inhibitor, 5-bromo-2′-deoxyuridine. L_6 myoblasts grown in the presence of anti-cadherin antibodies exhibited an altered morphology in comparison to control cultures, coupled with decreased myoblast fusion. These data indicate that the developmental regulation of cadherin is part of the program of terminal differentiation of skeletal myoblasts, and that cadherins are involved in the process of myoblast fusion.

5.2 Introduction

Skeletal myoblasts are embryonic muscle precursor cells which differentiate and ultimately undergo plasma membrane fusion to form syncytial cells called myotubes (for review, see Wakelam, 1985). Stable adhesive interactions must first be established between fusion-competent myoblasts in order for plasma membrane fusion to occur (Knudsen and Horwitz, 1978). A large increase in calcium-dependent adhesion is observed at the onset of fusion in embryonic chick myoblasts (Knudsen and Horwitz, 1977). Knudsen (1985) has shown that these initial calcium-dependent interactions are mediated by a glycoprotein which is protected from proteolysis by calcium. These

observations led us to speculate that a member of the cadherin family of calcium-dependent cell adhesion molecules could be mediating these adhesive interactions.

Cadherins mediate calcium-dependent intercellular adhesion by a homophilic mechanism (Takeichi, 1988; Nagafuchi et al., 1987). They are protected from proteolysis in the presence of calcium (Takeichi, 1977). Immunochemical methods suggest that a cadherin detected on the surface of muscle cells is the neural form of cadherin, N-cadherin (Hatta et al., 1987; Miyatani et al., 1989).

In this communication we demonstrate that a cadherin is expressed by skeletal myoblasts. The levels of this cadherin are developmentally regulated during the differentiation of L_6 myoblasts. Cultivation of the myoblasts in the presence of antibodies directed against cadherin altered cell morphology and decreased plasma membrane fusion. We suggest that a cadherin may mediate the calcium-dependent myoblast interactions which precede myoblast fusion.

5.3 Materials and methods

5.31 Cell cultures

The L_6-E9 myoblasts (Ball et al., 1979) used in these studies are a subclone of the L_6 line of immortalized rat skeletal myoblasts (Yaffe, 1968) and were obtained from Dr. B.D. Sanwal (University of Western Ontario). Myoblasts were grown in 140-mm culture dishes (Nunc, Burlington, Ont.; initial seeding density of 1.5×10^6 cells/dish) containing 24 ml of Dulbecco's Modified Essential Medium (DMEM) (GIBCO, Mississauga, Ont.) supplemented with 10% horse serum (GIBCO) (growth

medium) in an atmosphere containing 5% CO_2. In some experiments, the medium was replaced after 48 hr in culture with DMEM containing a 1:500 dilution of ITS supplement (insulin, transferrin, and selenium) (fusion medium) (Collaborative Research, Bedford, MA). Cells cultured in growth medium reached confluence after 3 days, with numerous fusion foci appearing on the following day. The extent of fusion was greater when cells were switched to fusion medium after 48 hr.When present, cells were treated with 6.5 μM 5-bromo-2′-deoxyuridine (BUdR) (Boehringer-Mannheim, Montreal, Que.). BUdR, when present, was added directly to the growth medium immediately after plating of the cells. This dose reliably decreases L_6 myoblast fusion to ~5% compared with untreated myoblasts. Morphological and fusion assays were performed in 96-well dishes. Cells were fixed with glutaraldehyde, extracted with methanol, and stained with 10% Harris hematoxylin followed by 1% eosin (Sigma, St. Louis, MO) (Lillie, 1954). The fusion index was determined by counting the number of nuclei in myotubes and dividing by the total number of nuclei (a myotube was defined as a cell containing a minimum of three nuclei). A total of 40 fields were examined for each point. Embryonic chick myoblasts were prepared as described previously (Charuk and Holland, 1983). Briefly, the pectoral muscle was dissected from 11-day old chick embryos and digested three times with 0.20% trypsin (Difco, Detroit, MI) in PBS. Cells were cultured in growth medium supplemented with 0.5% chicken embryo extract and seeded onto gelatinized 100-mm plastic dishes (Nunc). Under these conditions, embryonic chick myoblasts started to fuse by Day 2 postseeding and were virtually completely fused after 4 days in culture.

5.32 Preparation of anti-cadherin serum

The preparation and specificity of the antiserum (here designated CADH-1) has previously been described by Blaschuk and Farookhi (1989). CADH-1 was raised by immunizing rabbits with four synthetic peptides derived from avian N-cadherin. The sequences of these peptides are highly conserved among all cadherins (Hatta et al., 1987). Immunoglobulins were purified from CADH-1 and preimmune sera by affinity chromatography on a column containing protein A conjugated to Affi-Gel (Bio-Rad, Mississauga, Ont.) according to manufacturer's instructions.

5.33 Immunofluorescence

Cadherin was immunolocalized in L_6 myoblasts according to procedures previously described by Blaschuk and Farookhi (1989), with the exception that cells were not extracted with acetone. Briefly, cells were fixed with 3% formaldehyde for 10 min., washed with Tris-buffered saline (TBS) containing 0.1 *M* glycine, and sequentially probed with CADH-1 (diluted 1:25 in TBS + 50% goat serum) and goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate.

5.34 Sample preparation and immunoblot analysis

Total protein extracts were prepared from rat L_6 myoblasts, embryonic chick skeletal myoblasts and adult chicken brain as described by Blaschuk and Farookhi (1989). SDS-PAGE was performed according to the method of Laemmli (1970). Following electrophoresis, proteins were electroblotted according to the method of

Towbin et al. (1979). Blots were sequentially probed with either preimmune or CADH-1 sera diluted 1:100, followed by alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega, Madison, WI) as described by Blaschuk and Farookhi (1989). The DNA content of extracts was determined according to the procedures of Labarca and Paigen (1980), whereas the protein content of the extracts was assessed by the method of Bradford (1976).

5.4 Results and discussion

The observations presented by Knudsen and Horwitz (1977, 1978) demonstrate that intercellular adhesion between fusion-competent myoblasts is calcium-dependent. These observations led us to speculate that a cadherin may mediate this process. An avian cadherin, known variously as N-cadherin, N-cal-CAM, or A-CAM (M_r 127-135 kDa) is expressed in neural and muscle tissues, as well as a few epithelial tissues (Miyatani et al., 1989; Lagunowich and Grunwald, 1989; Volk and Geiger, 1986a,b). We have recently prepared and characterized an antiserum (CADH-1) capable of reacting with a variety of cadherins (Blaschuk and Farookhi, 1989). The CADH-1 antiserum was raised against synthetic peptides whose amino acid sequences are identical to those found in chicken N-cadherin. This antiserum is capable of reacting with N-cadherin from chicken brain extracts (M_r 127 kDa) (Fig. V:1, lane b). A single immunoreactive protein with a molecular mass similar to that of chicken N-cadherin was also detected in homogenates of chicken and rat L_6 myoblasts (Fig. V:1, lanes c, a).

We chose to concentrate our studies on rat L_6 myoblasts since these cells are clonally derived and their myogenesis has been extensively characterized (Yaffe, 1968; Florini and Magri, 1989). Immunofluorescence analysis of cadherin expression by aggregates of L_6 myoblasts and L_6 myotubes showed staining localized to the plasma membrane (Figs. V:2a, 2b).

Knowledge of the existence of a myoblast cadherin with properties similar to those of N-cadherin prompted us to investigate its developmental expression. Like other cadherins, the spatiotemporal expression of N-cadherin is tightly regulated during development and has been shown to correlate with increased cell adhesiveness (Hatta et al., 1987). We found that levels of cadherin in L_6 myoblasts increased during their differentiation into myotubes (Fig. V:3). Low levels of cadherin were observed in myoblasts after 24 hr. in culture (Fig. V:3, lane a). Cadherin levels increased ~20-fold in these cells after 72 hr in culture (Fig. V:3, lane c). At this stage, L_6 myoblasts are fusion-competent and myotubes are beginning to form. These observations indicate that cadherin levels are developmentally regulated during myogenesis *in vitro*. They also show that cadherin is maximally expressed as the myoblasts begin to adhere to one another prior to fusion.

It has been well established that myoblast differentiation and fusion can be reversibly inhibited by growing cells in medium containing the thymidine analog, BUdR, for the duration of culture (Stockdale et al., 1964; O'Neill and Stockdale, 1974). MyoD1 and myogenin have recently been identified and are thought to control commitment and differentiation in skeletal myoblasts by directly controlling muscle-

specific gene expression. The inhibition of myoblast differentiation by BUdR results from the suppression of MyoD1 and myogenin expressed when these genes are substituted with BUdR (Tapscott et al., 1989; Lin et al., 1989). However, levels of proteins unrelated to myogenesis, such as fibronectin, are not affected by BUdR (Holland et al., 1984). In view of these observations we examined the effect of BUdR upon cadherin expression in L_6 myoblasts. Cadherin levels in myoblasts grown for 3 days in BUdR-containing medium were ~threefold lower than levels in untreated, differentiation-competent cells (Fig. V:4). Levels of cadherin in BUdR-treated myoblasts remained unchanged after 7 days in culture (data not shown).

To evaluate the possible involvement of this cadherin in myoblast fusion, we examined the effect of purified CADH-1 Ig on myoblast morphology and fusion. Fusion-competent myoblasts exposed to CADH-1 Ig exhibited an altered morphology in comparison to cultures treated with preimmune Ig (Fig. V:5). In the presence of CADH-1 Ig, cells became elongated and detached from one another (Fig. V:5B, 5C). Cells exposed to preimmune Ig remained flattened and closely juxtaposed to each other (Fig. V:5A). In addition, the extent of fusion by myoblasts exposed to CADH-1 Ig decreased by 45% (preimmune = 54 ± 4%; CADH-1 = 29 ± 2%; Fig. V:6). Taken together, these observations suggest that a cadherin is involved in mediating the calcium-dependent interactions that occur between myoblasts prior to fusion (Knudsen and Horwitz, 1977, 1978). Knudsen et al., (1989) have also obtained preliminary results using chick myoblasts that are in agreement with this contention. We cannot as yet exclude the possibility that other cell adhesion molecules may also be involved in

mediating calcium-dependent myoblasts adhesion, as we have not determined the extent to which our antiserum can inhibit this process.

In summary, the data presented herein establish a correlation between an increased expression of cadherin and the program of differentiation of skeletal myoblasts. These data suggest that the cadherin gene expressed by myoblasts may be a novel member of the group of genes, such as muscle creatine phosphokinase (*mck*) and myosin heavy chain, which are induced during myoblast differentiation (Perriard, 1979; Devlin and Emerson, 1978; Hastings and Emerson, 1982).

Little is known regarding the mechanisms regulating cadherin expression. Since myogenesis is one of the best studied developmental processes and is easily manipulated *in vitro*, it constitutes an excellent system in which to study the regulation of cadherin expression during differentiation. We are now investigating the ability of growth and differentiation factors which influence myogenesis (e.g., transforming growth factor-β, myogenin) to regulate the expression of cadherin by myoblasts *in vitro*.

5.5 Acknowledgements

We thank Ms. R. Charbonneau for excellent technical assistance and Mr. C. Hodge and his colleagues for photographic services. This research was supported by grants from the Canadian Medical Research Council to O.W.B and P.C.H, as well as a grant from the Muscular Dystrophy Association of Canada to P.C.H.

5.6 References

References for this paper can be found in the reference section (Chapter 8).

Fig. V:1. Immunoblots of homogenates of fusion-competent L_6 myoblasts (A, D), adult chicken brain (B, E) and fusion-competent embryonic chick myoblasts (C, F). Blots were probed with either CADH-1 (lanes A-C), or preimmune serum (D, F). Molecular mass values (kDa) of a mixture of standard proteins electrophoresed along with the protein samples are shown on the left-hand side of the figure.

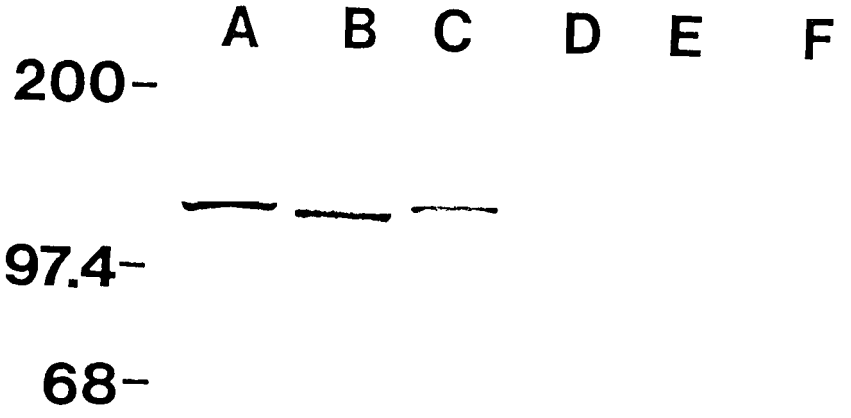

A B C D E F
200-
97.4-
68-

Fig. V:2. Immunofluorescence micrographs of L_6 myoblasts (A) and myotubes (B) probed with the CADH-1 antiserum. No fluorescence was observed when cells were probed with preimmune serum (not shown). (A) Bar = 4 μm. (B) Bar = 3 μm.

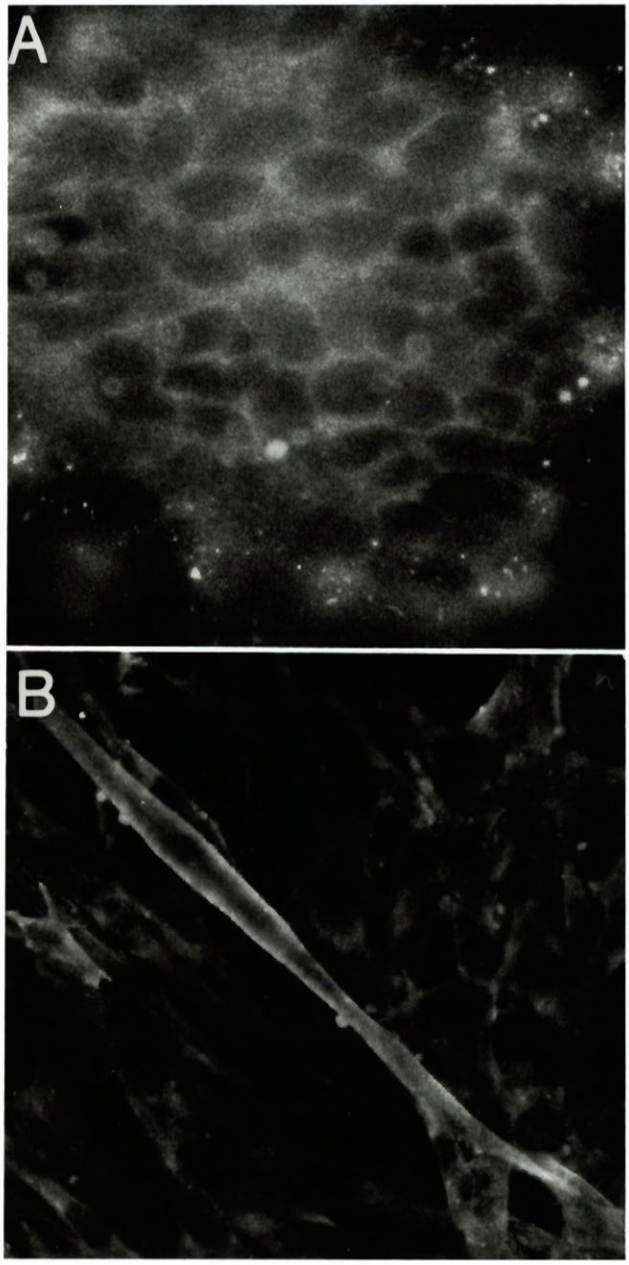
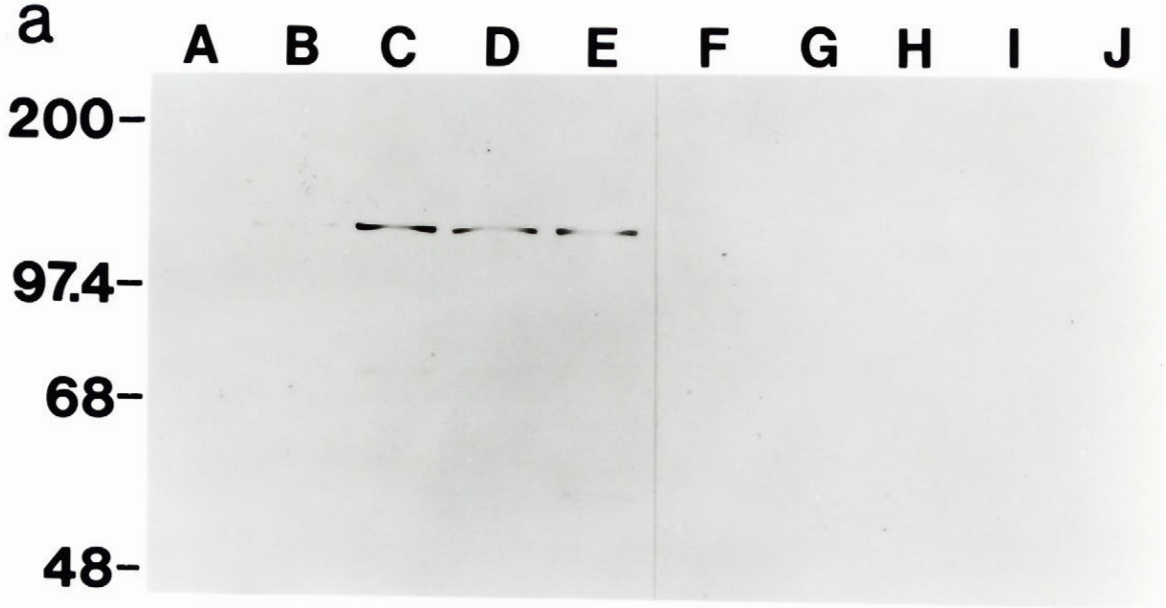

A
B

Fig. V:3. Expression of cadherin during L_6 myoblast differentiation. (a) Immunoblots of homogenates prepared from L_6 myoblasts cultured for 1 (A, F), 2 (B, G), 3 (C, H), 4 (D, I), and 6 (E, J) days. The blots were probed with either CADH-1 (A-E) or preimmune serum (F-J). Aliquots (each containing 1 μg of DNA) were taken from the samples and subjected to SDS-PAGE as described in the text. (b) Densitometric scans were performed on lanes A-E in order to quantitate levels of cadherin. Results are expressed as percentages of maximal induction.

a


A
B
C
D
E
F
G
H
I
J
200-
97.4-
68-
48-

b

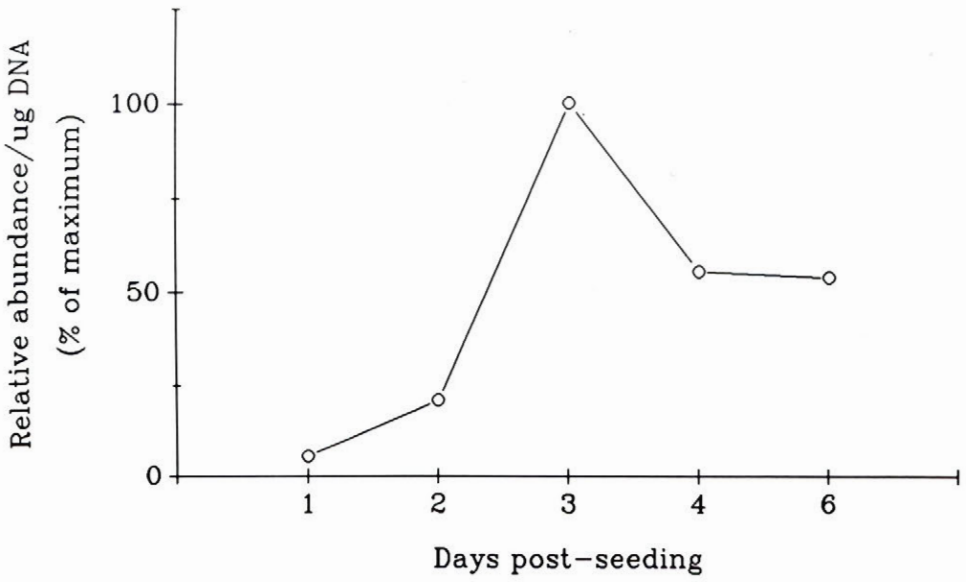

Relative abundance/ug DNA
(% of maximum)
100
50
0
1
2
3
4
6
Days post-seeding

Fig. V:4. Immunoblots of homogenates prepared from BUdR-treated (B, D) and untreated (A, C) L_6 myoblasts. The blots probed with either CADH-1 (A, B) or preimmune (C, D) serum. Aliquots containing 5 μg protein were analyzed.

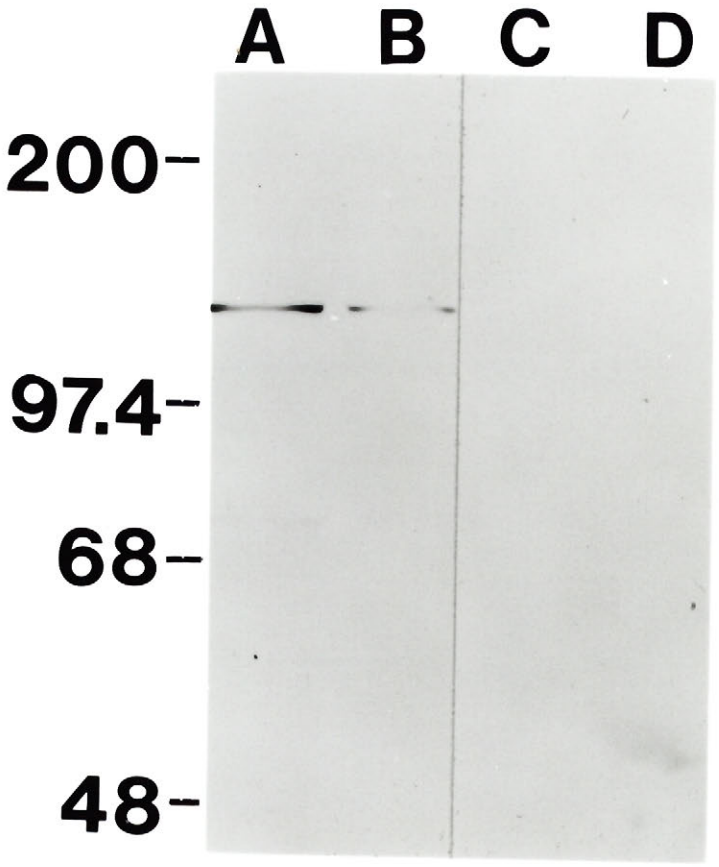

A
B
C
D
200-
97.4-
68-
48-

Fig. V:5. Effect of CADH-1 immunoglobulins on the morphology of fusion-competent L_6 myoblasts. Cells were exposed to either purified CADH-1 (B, C), or preimmune (A) Ig dissolved in fusion medium. Immunoglobulins (final concentration of 2 mg/ml)) were added to cells after 48 hr in culture. After 6 hr they were fixed and photographed. Bar = 5 μm.

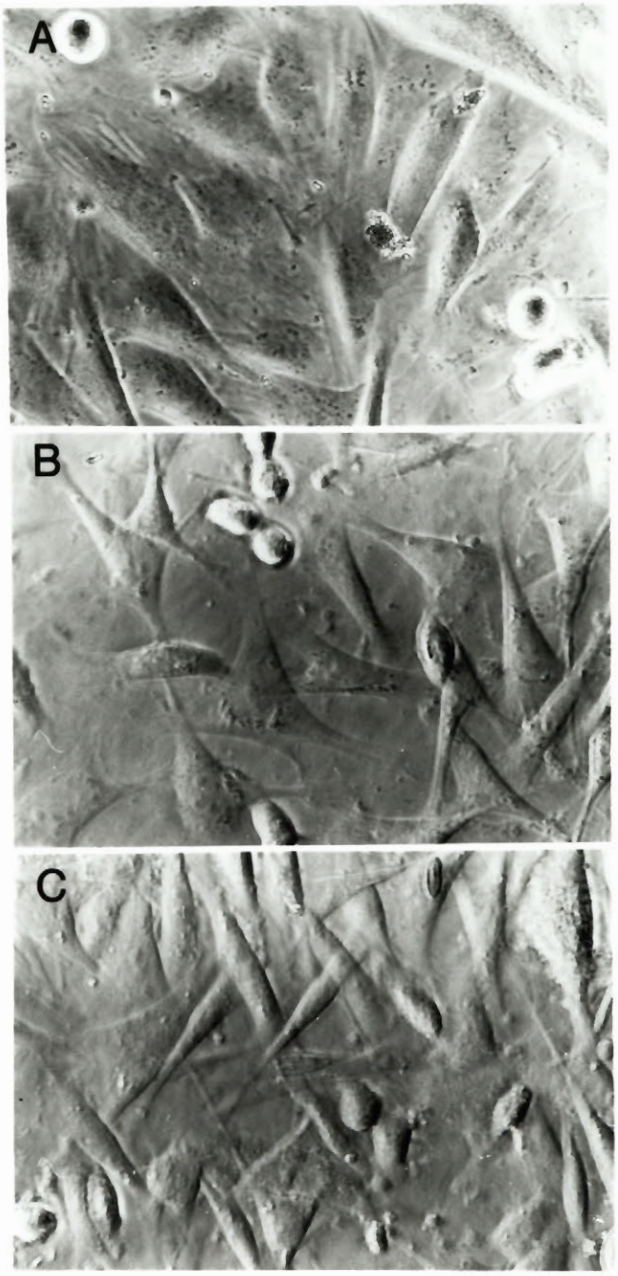
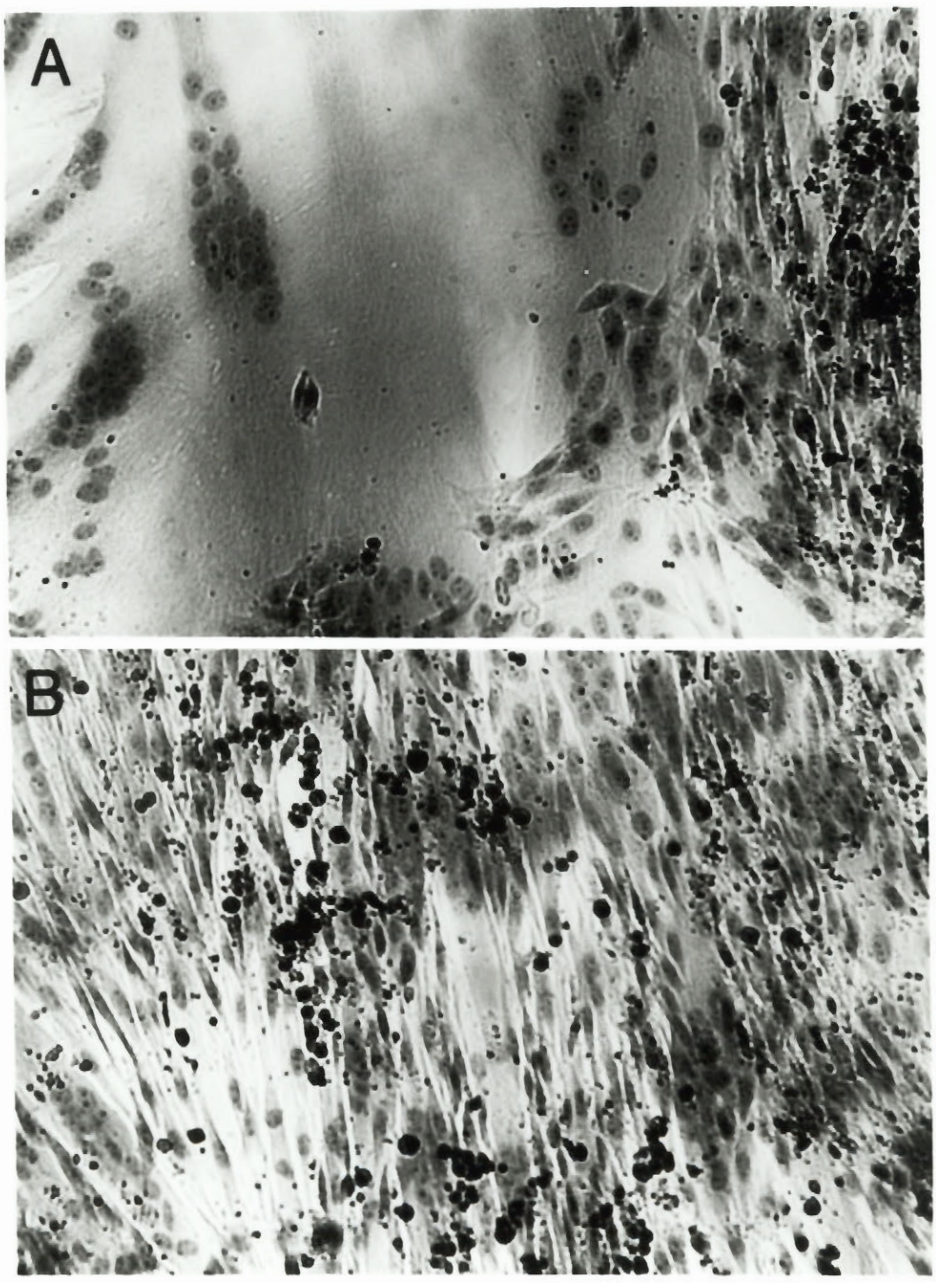

A
B
C

Fig. V:6. Effect of CADH-1 immunoglobulins on the fusion of L_6 myoblasts. Cells were exposed to either purified preimmune (A) or CADH-1 (B) Ig (final concentration of 0.5 mg/ml). Immunoglobulins were added to the growth medium after cells had been in culture for 48 hr. Cells were fixed and photographed 3 days after addition of the immunoglobulins. Bar = 15 μm.


A
B

6.0 DEVELOPMENTAL REGULATION OF M-CADHERIN IN THE TERMINAL DIFFERENTIATION OF SKELETAL MYOBLASTS[5]

FOREWORD

Following the immunological studies of cadherin expression in L_6 myoblasts described in Chapter 5, I proceeded to determine whether N-cadherin was expressed in these cells. At that time, N-cadherin was the only cadherin known to be expressed in mammalian myoblasts. While executing these studies, M-cadherin was isolated. I therefore performed a comparative analysis of the regulation of expression of both N- and M-cadherin during the differentiation of L_6 and C2 myoblasts. This analysis revealed that M-cadherin is regulated by the program of terminal myoblast differentiation.

6.1 Summary

Cadherins form a large family of membrane glycoproteins which mediate homophilic calcium-dependent cell adhesion. They are thought to mediate the initial calcium-dependent cell adhesion which precedes the plasma membrane fusion of skeletal myoblasts. Two cadherin subtypes are known to be expressed in mammalian skeletal myoblasts: muscle cadherin (M-cadherin) and neural cadherin (N-cadherin).

In the present study we demonstrate that (1) the expression of M- and N-cadherin is differentially regulated during myoblast differentiation in vitro, (2) that expression of M-cadherin but not N-cadherin is inhibited by BUdR, and (3) that fusion and differentiation-competent rat L_6 myoblasts do not express detectable levels of N-

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cadherin mRNA.

In vivo, M-cadherin mRNA was undetectable in adult rat tissues. In the embryo, M-cadherin mRNA was detectable exclusively in skeletal muscle. M-cadherin mRNA levels peaked during the secondary myogenic wave, becoming undetectable in one week-old neonates.

These observations indicate that M-cadherin is unique in two ways: it is the first cadherin to be included in the family of skeletal muscle-specific genes, and it is only the second cadherin known to be restricted to an embryonic tissue. Taken together, these results suggest that M-cadherin plays an important role in skeletal myogenesis.

6.2 Introduction

Cadherins constitute a family of multi-domain membrane glycoproteins of molecular mass ~120 kDa which mediate homophilic, calcium-dependent intercellular adhesion. Several subtypes with differing primary structures have been isolated (reviewed in Grunwald, 1993; Geiger & Ayalon, 1992; Pouliot, 1992). Individual subtypes are most likely encoded by single-copy genes which are controlled in a tissue- and developmental stage-specific fashion. Cadherins are thought to play an important role in morphogenesis by mediating specific intercellular adhesion which leads to cell sorting (reviewed in Takeichi, 1991, 1990).

Skeletal myoblasts are embryonic muscle precursor cells which differentiate and eventually undergo plasma membrane fusion to form syncytial cells called myotubes

(reviewed in Wakelam, 1985). A first step in the process of plasma membrane fusion is the establishment of stable adhesive interactions between fusion-competent myoblasts in order for fusion to occur (Knudsen & Horwitz, 1978; reviewed in Knudsen, 1990a). We (Pouliot et al., 1990a) and others (Knudsen et al., 1990b; Mege et al., 1992) have investigated the role of cadherins in the establishment of these interactions.

Several distinct cadherins have been identified in avian skeletal muscle or in avian myoblasts, namely N-cadherin (Hatta et al., 1987; Knudsen et al., 1990b), B-cadherin (Napolitano et al., 1991), R-cadherin (Inuzuka et al., 1991a) and T-cadherin (Ranscht & Zimmerman, 1991). In mammalian skeletal muscle cells, three cadherins have been identified to date, namely N-cadherin (Walsh et al., 1990), M-cadherin (Donalies et al., 1991) and R-cadherin (Hutton et al., 1993). It is not known whether homologs of B- or T-cadherin are expressed in mammalian skeletal muscle cells, whether R-cadherin is expressed in mammalian myoblasts, or whether a homolog of M-cadherin is expressed in avian skeletal muscle cells.

To date, functional studies on the possible role of individual cadherin subtypes in skeletal myogenesis have been limited to analyses of the role of N-cadherin in avian myoblast adhesion and fusion (Knudsen et al., 1990b; Mege et al., 1992). From these studies there is considerable evidence supporting a role for N-cadherin in the control of avian myoblast fusion.

Our own earlier studies in mammalian myoblasts (Pouliot et al., 1990a) utilised an antiserum directed against synthetic peptides corresponding to amino acid sequences

which are highly conserved in the known cadherins. Consequently, this antiserum could not identify individual cadherin subtypes. However, we demonstrated (Pouliot et al., 1990a) that (1) a cadherin expressed in L_6 myoblasts is developmentally regulated during myoblast terminal differentiation; (2) that antibodies directed against conserved sequences in the extracellular domain of cadherin interfere with L_6 myoblast fusion, and (3) that L_6 cadherin levels are significantly depressed by 5-bromo-2'-deoxyuridine (BUdR), an agent which inhibits skeletal myoblast differentiation (Stockdale et al., 1964).

Here we show that differentiation-competent L_6 myoblasts express M-cadherin but do not express detectable amounts of N-cadherin mRNA. We further show that in the mouse C2 myoblast cell line, which expresses both M- and N-cadherin, these two cadherins are differentially regulated. The susceptibility of M-cadherin to BUdR, coupled with its pattern of expression restricted to developing skeletal muscle, suggests a role for M-cadherin in myoblast differentiation.

6.3 Methods

6.31 RNA extraction

For cells grown in culture, monolayers were washed three times in PBS and homogenized directly in extraction buffer (0.5% SDS/50 mM Tris-HCl (pH 7.5)/1 mM EDTA), followed by two extractions with an equal volume of water-saturated phenol and one extraction with chloroform. The aqueous phase was then precipitated overnight at -70°C in 2.5 volumes of ice-cold ethanol. The pellet was resuspended in TE and

RNA precipitated overnight in 2 M LiCl at 4°C according to the method of Sambrook et al (1989). The precipitate was then spun at 3000 X g for 20 min and resuspended in water. Tissues were pulverized in liquid nitrogen and extracted using the same method, with the exception that additional cycles of phenol extraction and ethanol-precipitation were used to remove excess impurities.

For embryonic skeletal muscle, hind limbs were placed in extraction buffer, disrupted with either a Dounce glass-teflon homogenizer or a Polytron homogenizer (Brinkman instruments) and total RNA prepared by phenol/chloroform extraction as described above.

Poly(A)$^+$ RNA was prepared using either the Poly(A) Quik kit from Stratagene (for large-scale preparations) or, for small-scale preparations, using the Micro-Fast Track mRNA isolation kit from InVitrogen. Manufacturer's instructions were followed in both cases.

6.32 Northern analysis

RNA samples were separated by gel electrophoresis in 1.5 % agarose gels containing 7% formaldehyde using MOPS running buffer (Sambrook et al, 1989). Gels were transferred onto Zetaprobe-GT membranes (BioRad) by vacuum blotting, followed by cross-linking of the blot under UV (Stratagene). Blots were pre-incubated at 42°C for two hours in hybridization buffer (50% formamide, 5X SSPE, 5X Denhardt's, 10% dextran sulfate, 100 µg/ml ssDNA, 1% SDS), and then hybridized overnight at 42°C. Probes were labelled with 5'-[α^{32}P]-dCTP (Amersham) using a

random-priming kit (Pharmacia) as per manufacturer's instructions. Following probing, blots were rinsed several times with 2X SSPE, twice for 20 minutes each at room temperature with 2X SSPE + 0.1% SDS, and twice for 40 min each with 0.1X SSPE + 0.1% SDS at 65°C.

For quantitation, blots were exposed to an imaging plate of the PhosphorImager system (Molecular Dynamics). Bands were quantitated using the ImageQuant software provided with the system (version 3.15). Variations in amounts of RNA loaded were usually normalized against 18S ribosomal RNA, except where otherwise indicated. For fluorography, Kodak XAR film was exposed for ~ 2 days at -70°C between two intensifying screens.

6.33 cDNA probes

The mouse M-cadherin cDNA was provided courtesy of Dr. A. Starzinski-Powitz (Universität zu Köln) (Donalies et al., 1991). A BamHI 978 base-pair fragment encoding the fifth extracellular domain, the transmembrane region and approximately half of the cytoplasmic region of M-cadherin was used to probe Northern blots. The mouse N-cadherin cDNA used here (a gift from M. Takeichi, Kyoto University) is ~ 1 kb-long and encodes the N-terminal region of the protein. It is identical to the mn-2 probe described in Miyatani et al., (1989).

Rat fast troponin I (fast TnI) was obtained from K. E. M. Hastings and M. Gravel (McGill University, unpublished; GENBANK accession number M73701). Plasmid pUC65-2, containing the complete coding sequence for rat myogenin, was

provided courtesy of Dr. W. E. Wright (University of Texas) (Wright et al., 1989). The cDNA encoding the rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was originally sent by Dr. B. Murphy (SRI International) to Dr. E. Shoubridge (McGill University), while plasmid pHMαA-1 encoding the LK248 actin sequence was provided by Dr. L. Kedes (Veterans Administration, Palo Alto). For these cDNAS, complete, linearized plasmids were used to generate probes.

6.34 Protein sample preparation and immunoblotting

Total protein extracts of L_6 myoblasts were prepared, electrophoresed by SDS-PAGE (Laemmli, 1970) and immunoblotted as described previously (Pouliot et al., 1990a), except that blots were blocked using a tris-buffered saline solution containing 2% skim milk. Blots were probed with the affinity-purified anti-M-cadherin polyclonal antiserum, sc-69 (Santa Cruz Biotechnology, USA), and developed using the ECL chemiluminescent system (Amersham, Canada) as per manufacturer's instructions.

6.35 Cell culture and rodent tissues

The L_6-E9 myoblasts (Ball et al., 1979) used in these studies are a subclone of the L_6 line of immortalized rat skeletal myoblasts (Yaffe, 1968) and were obtained from Dr. B. D. Sanwal (University of Western Ontario). Myoblasts were grown aneurally in 140 mm Petri dishes (Nunc, Burlington, Ont.) at a seeding density of 10 X $10^3/cm^2$. During the first 72 hours in culture, cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum

(FBS) (growth medium) in an atmosphere containing 5% CO_2. After 72 hours, the medium was replaced with DMEM supplemented with insulin, transferrin and selenium (differentiation medium). Under these conditions, large-scale fusion can be observed 2-3 days after the switch (day 6 in culture).

The C2 line of immortalized mouse skeletal myoblasts (Yaffe and Saxel, 1977) were obtained from B. Paterson (NIH). A subclone selected for high fusion index was used for these studies. Myoblasts were grown aneurally in growth medium consisting of DMEM + 20% FBS (growth medium), and induced to differentiate by transfer to DMEM + 2% horse serum. Extensive fusion was observed three days after the substitution to differentiation medium (day 6 in culture).

For both L_6 and C2 myoblasts, treatment with 6.5 μM BUdR was performed as described previously (Pouliot et al., 1990a) by the addition of the drug directly to the growth medium immediately after plating of the cells. This dose reproducibly decreased the fusion index (number of nuclei inside cells with ≥3 nuclei/total number of nuclei) of L_6 and C2 myoblast to <5% of the level observed in untreated myoblasts.

Rat primary myoblasts were prepared as described previously (Holland and MacLennan, 1976). Briefly, 2-day old Sprague-Dawley neonates were decapitated and their hind limb muscles removed, minced in PBS, and repeatedly trypsinized using PBS containing 0.3% crude trypsin at 37°C until complete digestion of the muscle. Following neutralization of the trypsin using excess medium, cells were pelleted, counted, and plated at 2 X 10^6 cells/100 mm dish. Pre-plating onto plastic was used to enhance for myogenic cells (Holland and MacLennan, 1976). Cells were grown in

DMEM + 10% FBS + 2% chicken embryo extract (growth medium). Differentiation of myoblasts was induced after 2-3 days in culture by diluting the growth medium five-fold with DMEM in order to decrease the concentration of growth factors. Numerous myotubes were observed after 2-3 days, at which point the cells were harvested. Mouse primary myoblasts from 2-day old CD1 neonates were prepared in the same way.

For the isolation of embryonic skeletal muscle RNA, time pregnant Sprague-Dawley rats were obtained from Charles River (Canada). Embryos were taken at ED14, ED15, ED16, ED18, while postnatal animals were used 1 day and 1 week after birth. Dissection of hind limb muscles was performed under a binocular microscope. For all of stages, the hind limbs were dissected from the trunk at the thigh and the feet removed. Skin was removed from ED18 hind limb embryos and postnatal rats.

Other tissues from adult or E18 rats were obtained by sacrificing either 120-old day rats or timed-pregnant Sprague-Dawley rats, respectively, dissecting their respective tissues and isolating either total cell RNA (adults) or poly(A)$^+$ RNA (embryos) using the Micro-Fast Track mRNA isolation kit.

6.4 Results

6.41 N-cadherin mRNA is detectable in C2 cells but not in L_6 cells

The steady-state levels of N- and M-cadherin mRNA in mouse C2 and rat L_6 myoblasts were investigated by Northern blot hybridization using cDNAs encoding the mouse homolog of these cadherins. In C2 myoblasts, the N-cadherin probe hybridized

with two mRNA species (Fig. VI:1, panel A, lanes 7-8) which co-migrated with mRNAs present in adult rat heart (lane 1), a tissue known to express N-cadherin in mouse (Miyatani et al., 1989). In contrast, N-cadherin mRNAs were undetectable in total or poly(A)$^+$ RNA from L_6 myoblasts taken at several points during differentiation (Fig. VI:1, lanes 2-6, 9, respectively). Although not detectable in L_6 cells, N-cadherin mRNA was present in primary rat myotubes as well as in primary mouse myotubes, where it is coexpressed with M-cadherin (Fig. VI:2, lanes 4 and 2, respectively).

When the blot depicted in Fig. VI:1 was re-probed using the M-cadherin probe, a strong signal was observed in both C2 and L_6 myoblasts (Fig. VI:1, panel B, lanes 2-9). No signal was detected in adult rat heart. In addition to a predominant transcript of ~ 3.4 kb, a second, larger mRNA was detected in L_6 myoblasts (lanes 2-6, lane 9). The intensity of this transcript consistently mirrored that of the smaller size mRNA. However, the larger transcript could not be detected in mouse C2 myoblast or myotubes (Fig. VI:1, panel B, lanes 7, 8). Since the smaller mRNA found in L_6 myoblasts co-migrates with the M-cadherin mRNA in C2 myoblasts (from which M-cadherin was cloned), we will refer to this mRNA as the rat M-cadherin mRNA. The identity of the larger mRNA found in L_6 myoblasts and in embryonic rat muscle (see below) remains to be determined.

6.42 M-cadherin mRNA levels are developmentally regulated during the differentiation of L_6 myoblasts

Levels of M-cadherin mRNA were low in actively dividing L_6 myoblasts, but

transferring the cells to differentiation medium (i.e., mitogen-poor medium) resulted in increased mRNA levels, reaching a maximal level 24 hours after transfer (Fig. VI:3). This peak was followed by a decrease extending over several days. A similar pattern was also observed with myogenin mRNA (Fig. VI:3). The induction of M-cadherin mRNA accumulation in L_6 cells was also similar to that of fast TnI mRNA, a muscle-specific, myogenin-dependent gene (Lin et al., 1991). High levels of TnI mRNA are expressed following the onset of differentiation in primary rat myoblasts (Hinterberger et al., 1991), C2 and L_6 myoblasts (Koppe et al., 1989). In L_6 myoblasts, the levels of fast TnI transcripts increased during differentiation by approximately the same extent as myogenin and M-cadherin transcripts, although TnI message levels did not decline at later stages of culture as do levels of myogenin and M-cadherin mRNA (Fig. VI:3).

6.43 Expression of M-cadherin mRNA *in vivo* is highly restricted

Several embryonic tissues were probed for the presence of M-cadherin mRNA. Northern analysis detected M-cadherin transcripts only in skeletal muscle (Fig. VI:4, lane 2). In these muscles, M-cadherin transcripts were barely detectable in E14 embryos. However, levels increased rapidly, reaching a maximum by E18 (Fig. VI:5). M-cadherin mRNA levels then decreased in neonates, and transcripts were undetectable in one-week old or adult animals. Adult liver, gastrocnemius, heart (Fig. VI:6, panel A, lanes 10-12, respectively), as well as brain, skin and placenta (not shown) also failed to reveal M-cadherin mRNA. M-cadherin expression is thus limited to an early phase of skeletal myogenesis. In comparison, fast TnI mRNA began to accumulate somewhat

later in development, reaching a maximum in neonates and remaining high in the adult muscle (Fig. VI:5, panel B), as previously reported (Sutherland et al., 1991).

6.44 M-cadherin expression is BUdR-sensitive

The temporal pattern of developmental regulation of M-cadherin expression suggests it may be controlled by the skeletal myoblast differentiation program. We therefore investigated the effect of BUdR on M-cadherin expression. BUdR selectively inhibits terminal myoblast differentiation (Stockdale et al., 1964) by suppressing expression of helix-loop-helix muscle determination genes (Tapscott et al., 1989). Steady-state levels of M-cadherin mRNA in BUdR-treated L_6 myoblasts were markedly decreased when compared to levels in untreated cells (Fig. VI:6, panel A, compare lanes 3 & 5 with lanes 2 & 4). A similar decrease in steady-state levels of M-cadherin mRNA levels was seen in BUdR-treated C2 myoblasts (Fig. VI:6, panel A, compare lanes 6 & 8 with lanes 7 & 9). This effect was also observed at the protein level, as Western blot analysis showed a strong decrease in M-cadherin protein in BUdR-treated L_6 and C2 cells (Fig. VI:7).

In marked contrast to the strong effect of BUdR upon M-cadherin mRNA levels observed in both cell lines, the levels of N-cadherin mRNA were not depressed in BUdR-treated C2 cells (Fig. VI:6, panel B).

6.5 Discussion

Our previous study of the role of cadherin in the fusion and differentiation of

L_6 myoblasts indicated that these cells express a BUdR-sensitive, developmentally-regulated cadherin. Addition of purified anti-cadherin immunoglobulins to the culture medium significantly reduced plasma membrane fusion, suggesting that this cadherin was involved in myoblast fusion (Pouliot et al., 1990a). However, the identity of this cadherin was not determined. It was subsequently shown that two cadherins are expressed in the C2 line of mouse myoblasts: N-cadherin (Pouliot et al., 1990b; Walsh et al., 1990) and M-cadherin (Donalies et al., 1991).

In the present study we show that M-cadherin but not N-cadherin is expressed in L_6 myoblasts, and is induced during differentiation in vitro. Furthermore, levels of M-cadherin mRNA transcripts and M-cadherin protein in both C2 and L_6 cells were strongly decreased by BUdR. In contrast, N-cadherin expression was essentially unaffected by BUdR treatment. The pattern of expression of M-cadherin, taken together with its BUdR-sensitivity and the lack of detectable N-cadherin expression in L_6 myoblasts, suggest that the cadherin previously detected in L_6 and implicated in the control of myoblast fusion (Pouliot et al., 1990a) is M-cadherin.

It is of particular interest that we could not detect N-cadherin transcripts in the rat L_6 myoblast line. This result was further substantiated by sequencing several cDNA clones obtained by oligo-(dT)-primed reverse transcription coupled to the polymerase chain reaction (RT-PCR) using N-cadherin specific primers. No cadherins other than M-cadherin could be identified in L_6 cells (data not shown). Since N-cadherin transcripts can be detected in both mouse and rat myoblast primary cultures, the gene for N-cadherin appears to have been lost or become defective in the L_6 line.

Although there is as yet no direct evidence for a role for N-cadherin in mammalian myoblast fusion, there is considerable evidence for a role in the control of chick myoblast fusion. Anti-N-cadherin antibodies can interfere with the calcium-dependent cell aggregation of chick myoblasts (Knudsen et al., 1990c), as well as block myoblast fusion (Mege et al., 1992). Thus, when present, N-cadherin may also play a permissive role in the fusion of rat myoblasts.

The different temporal regulation of M- and N-cadherin expression during myogenesis, as well as their different response to inhibition of myoblast terminal differentiation by BUdR, suggest that these cadherins may play different roles in the formation of skeletal muscle. Recent experiments (Hahn and Covault, 1992) in chicks indicate a role for N-cadherin in nerve-muscle interactions, rather than in myoblast-myoblast interactions. Alternatively, Donalies et al. (1991) have speculated that N-cadherin might be involved in the self-recognition of myoblasts rather than in providing a triggering signal for plasma membrane fusion. According to this hypothesis, N-cadherin would play a facilitatory role whereas M-cadherin might mediate the trigger function leading to fusion.

Whereas N-cadherin is expressed in several embryonic and adult tissues, M-cadherin mRNA was expressed exclusively in embryonic skeletal muscle and in myoblasts/myotubes, and was otherwise undetectable in any other cell types of the embryo or the adult. Of the cadherins so isolated so far, M-cadherin is the only skeletal muscle-specific cadherin, and joins B-cadherin (Napolitano et al., 1991) as the only other cadherin expressed solely in the embryo.

Moore and Walsh (1993) have recently described a study of M-cadherin expression during mouse embryogenesis. Results from that study, which used in situ hybridization rather than quantitative Northern analysis, are in complete agreement with the data presented here, with the exception that M-cadherin-positive cells were detected earlier, at E8.5 (Moore et al., 1993). In the present study, we have also shown that there is a distinct peak of M-cadherin expression both in vivo and in vitro, and that M-cadherin expression is inhibited by treatment with BUdR.

The data presented here do not suggest post-transcriptional regulation of M-cadherin expression such as observed for B-cadherin expression (Napolitano et al., 1991). Rather, the regulation of M-cadherin expression during skeletal myogenesis appears to occur at the transcriptional level, as variations in M-cadherin mRNA levels are mirrored at the protein level in both C2 and L_6 cells (Fig. VI:7). The inhibition of M-cadherin transcription by BUdR (Fig. VI:7) further suggests the possibility that the regulation of M-cadherin expression during myogenesis is under the control of members of the helix-loop-helix family of genes.

Multiple stages can be defined during the formation of skeletal muscle tissue. These include the determination of cells in the myogenic lineage, the proliferation and alignment of myoblasts, their terminal differentiation and fusion to form multinucleated myotubes, and finally myotube innervation and further differentiation into specific muscle fibre types. At least two cadherin genes are expressed in developing murine skeletal myoblasts. The present study indicates that the expression of one of these, M-cadherin, is closely associated with the terminal differentiation of L_6 myoblasts, and

suggests a role in myoblast differentiation.

6.6 Acknowledgements

We would like to thank Mr. Claude Guérin for excellent technical assistance, Drs Hastings, Kedes, Murphy, Starzinski-Powitz, Takeichi and Wright for providing the various cDNA clones used here, Dr. C. Auffray for use of sequencing facilities, as well as C. P. Hodge for photographic work. This work was supported by a graduate fellowship to Y.P. from the Fonds de la Recherche en Santé du Québec. P.C.H. is the recipient of a grant from the Medical Research Council of Canada. M.G. was supported by a post-doctoral fellowship from the Fonds de la Recherche en Santé du Québec.

6.7 References

References for this paper can be found in the reference section (Chapter 8).

Fig. VI:1. Differential expression of N- and M-cadherin transcripts in C2 and L_6 myoblasts

Total and poly(A)$^+$ RNA was electrophoresed, blotted and probed sequentially with N-cadherin (panel A) and M-cadherin probes (panel B) as described in the Methods sections. Both blots were exposed for ~ 2 days. Panel C shows the 18S ribosomal RNA to indicate the corresponding loading in the ethidium-bromide stained gel.

Lanes 1-7: total RNA. Lanes 8-9: poly(A)$^+$ RNA. Lane 1: adult rat heart. Lanes 2-4: L_6 myoblasts extracted on days 2, 4 and 6 of culture, respectively. Lane 5: L_6 myoblasts extracted on day 3. Lane 6: L_6 myoblasts treated with 6.5 μM BUdR from the time of plating and extracted on day 3. Lane 7-8: C2 myoblasts extracted on day 7. Lane 9: L_6 myoblasts extracted on day 4. A 7-day over-exposure of the blot depicted in panel (A) failed to reveal any signal for N-cadherin mRNA in L_6 myoblasts. Molecular sizes for these transcripts are indicated in Fig. VI:4.

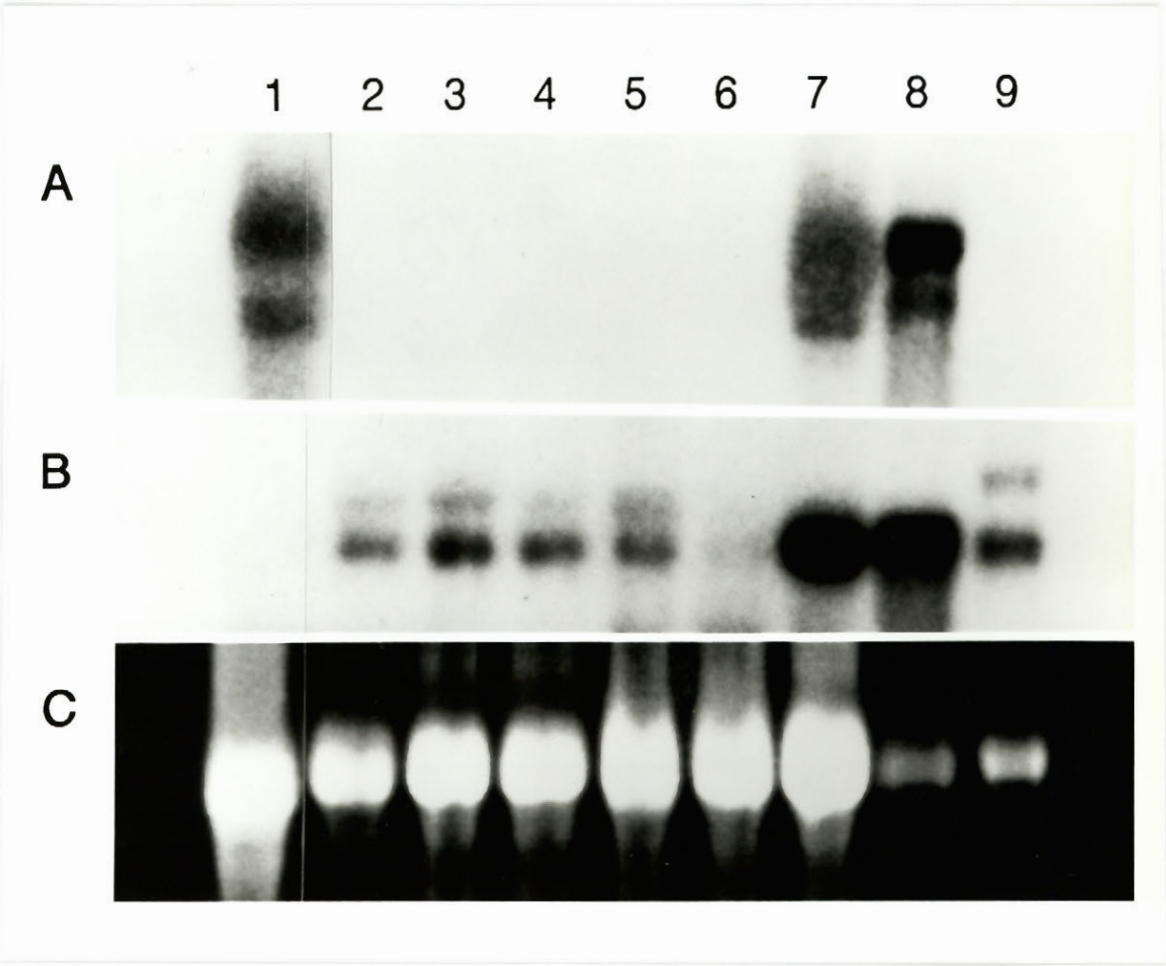

1 2 3 4 5 6 7 8 9
A
B
C

Fig. VI:2. N-cadherin mRNAs are detectable in rat primary myoblast cultures

Poly(A)$^+$ RNA was extracted from cultures of the C2 and L_6 muscle cell lines, and from mouse and rat primary myotubes. The blot was probed sequentially with the N-cadherin (panel A) and M-cadherin probes (panel B) and exposed to a phosphor imaging plate. The position of the 28S ribosomal subunit from whole cell RNA used as marker (not shown) is indicated.

Lane 1: C2 myoblasts; lane 2: mouse primary myotubes; lane 3: L_6 myoblasts; lane 4: rat primary myotubes.

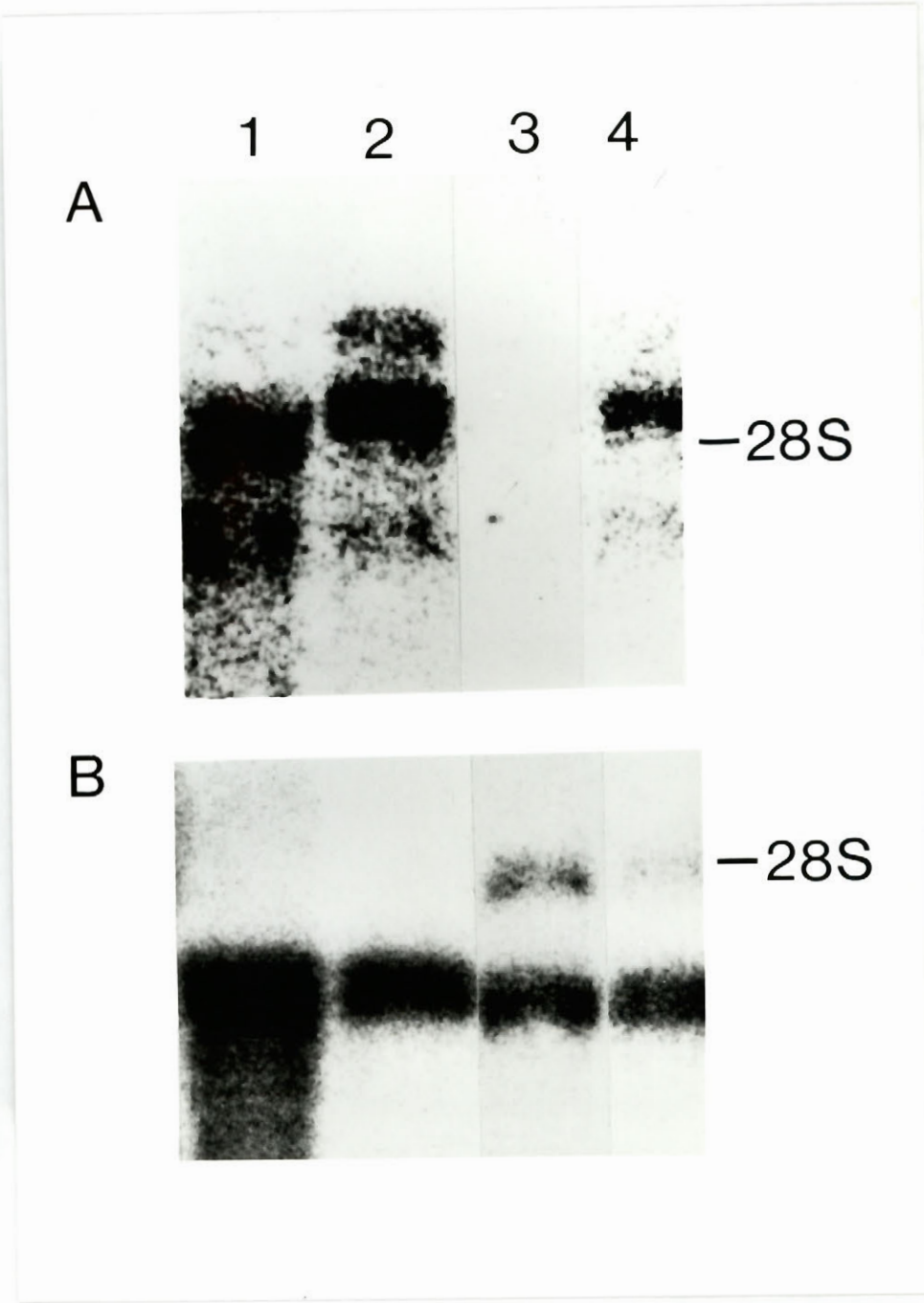

1
2
3
4
A
—28S
B
—28S

Fig. VI:3. The expression pattern of M-cadherin mRNA in L6 myoblasts mirrors that of muscle-specific genes

Cells were grown for the durations indicated in the legend, after which total RNA was extracted and probed sequentially with M-cadherin, rat fast TnI and rat myogenin cDNA by Northern blotting. The relative amounts of mRNA were determined by autoradiography and densitometry. All values were normalized to 18S ribosomal RNA and expressed as percent of maximum induction. For M-cadherin mRNA, only the 3.4 kb transcript was quantitated. Combined results from two experiments are shown.

Open circles: M-cadherin mRNA; closed circles: myogenin mRNA; open triangles: rat fast TnI mRNA.

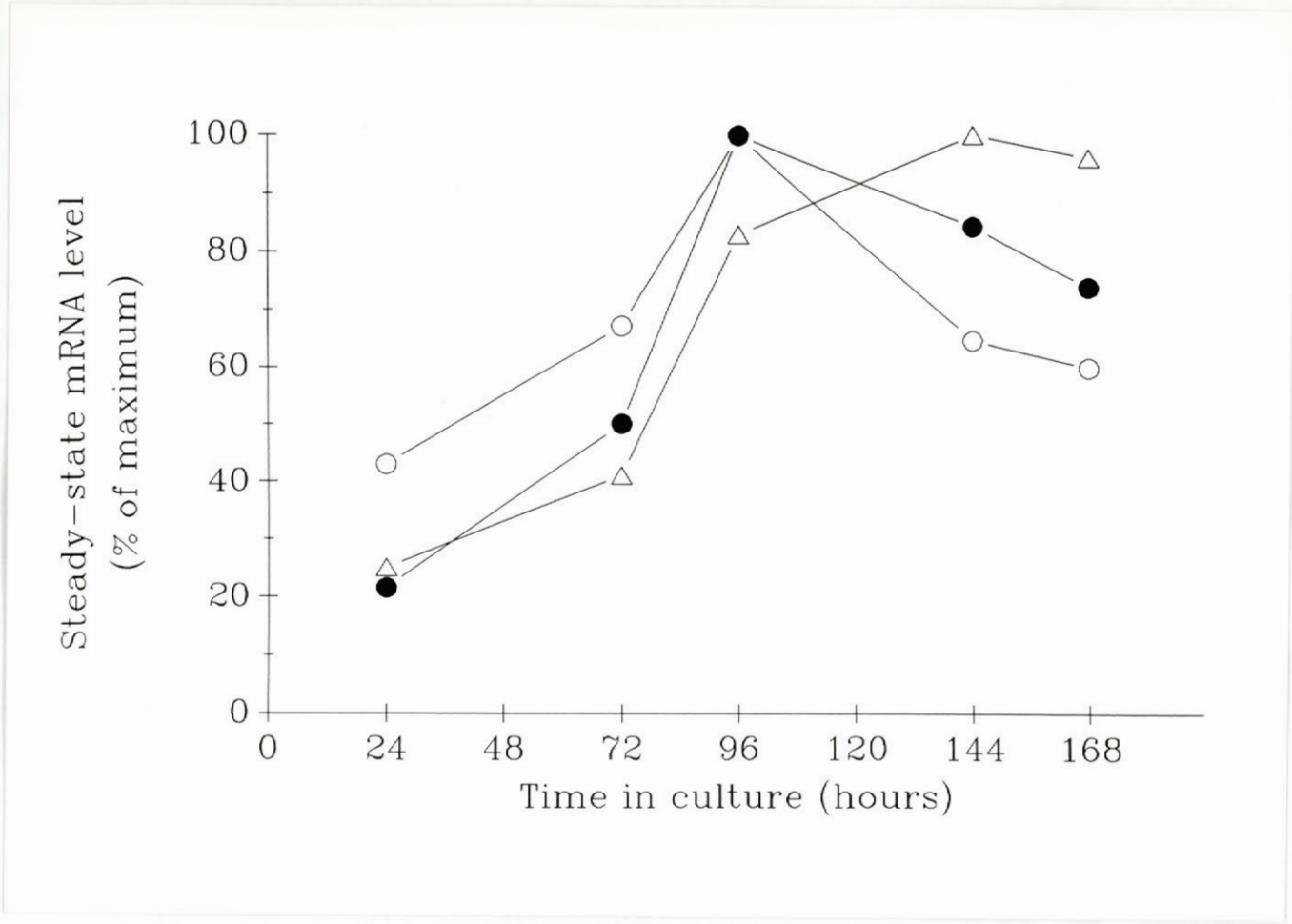

Steady-state mRNA level
(% of maximum)
100
80
60
40
20
0
0
24
48
72
96
120
144
168
Time in culture (hours)

Fig. VI:4. M-cadherin transcripts are undetectable in embryonic tissues other than skeletal muscle

Tissues from E18 rat embryos were dissected and poly(A)$^+$ RNA isolated. The blot was probed sequentially with the M-cadherin (Panel A) and GAPDH cDNAs (panel B).

Lane 1: total cell RNA from C2 myoblasts extracted on day 3. Lane 2: poly(A)$^+$ RNA extracted from primary rat myoblast/myotube cultures derived from neonatal quadriceps. Lanes 3-5: poly(A)$^+$ RNA from E18 rat brain, heart and liver, respectively.

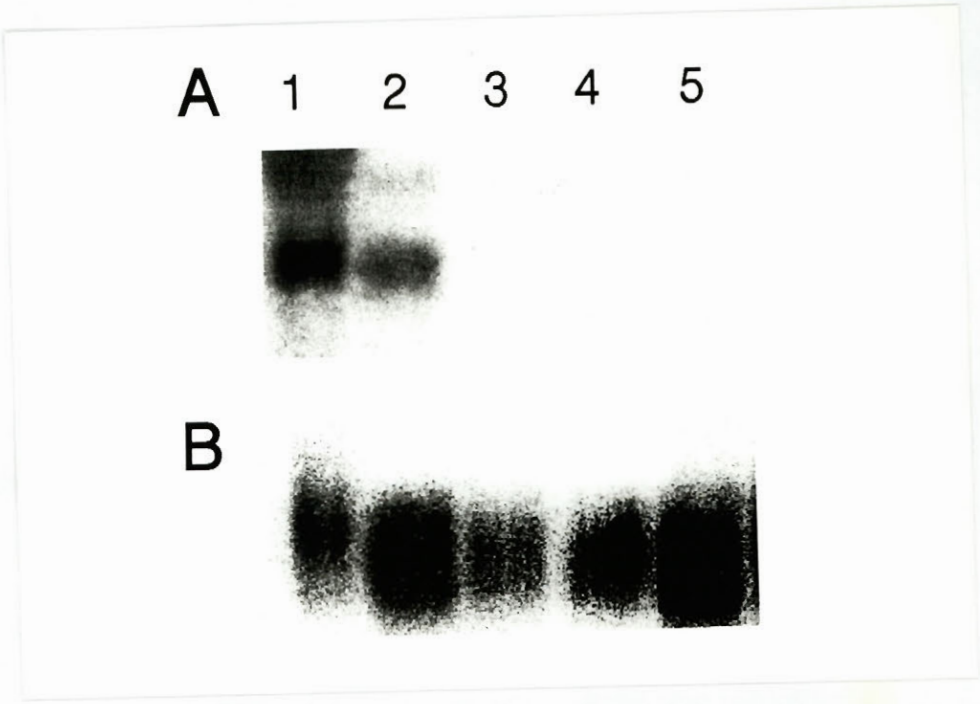

A
1
2
3
4
5
B

Fig VI:5. M-cadherin mRNA levels are regulated during embryonic rat myogenesis

Total cell RNA was isolated from embryonic rat hind limbs, as well as C2 and adult tissues. The blot was probed sequentially with the M-cadherin (Panel A), GAPDH (not shown) and fast TnI cDNAs (not shown). Following autoradiography onto a phosphor imaging plate, bands were quantitated using the ImageQuant software. Panel B shows relative levels of transcripts for M-cadherin (filled bars) and fast TnI (open bars). Levels are normalized relative to the level of GAPDH mRNA in each sample and are expressed as a percentage of each transcript's maximal level. Levels of M-cadherin and fast TnI for adult soleus are included for comparison with embryonic tissues.

Lane 1: total cell RNA from C2 myoblasts extracted on day 3. Lanes 2-5: total cell RNA extracted from embryonic hind limb muscles dissected on E14, E15, E16, E18, respectively. Lanes 6-7: total cell RNA extracted from hind limb muscle of neonatal and 1-week old animals, respectively. Lanes 8-11: total cell RNA extracted from adult rat soleus muscle, gastrocnemius muscle, liver; heart, respectively.

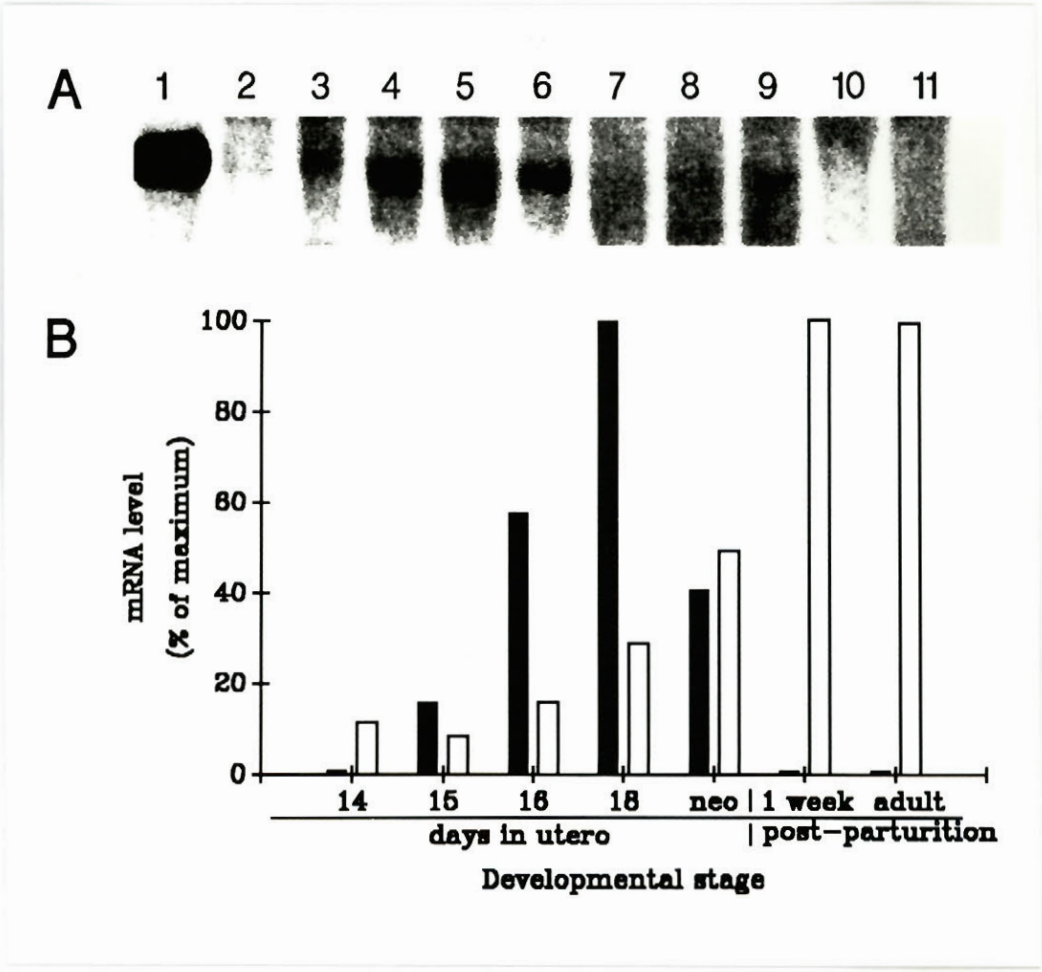

A
1
2
3
4
5
6
7
8
9
10
11
B
100
80
60
40
20
0
mRNA level
(% of maximum)
14
15
16
18
neo
1 week
adult
days in utero
post-parturition
Developmental stage

Fig. VI:6. BUdR suppresses the expression of M-cadherin mRNA in C2 and L_6 myoblasts

Cells were grown in the presence or absence of 6.5 μM BUdR for the durations indicated in the legend, after which total RNA was extracted. Blots were probed sequentially with M-cadherin (panel A) and N-cadherin probes (panel B) according to the method described above. Panel C shows the 18S ribosomal RNA. In addition to the extensive inhibition of plasma membrane fusion observed in BUdR-treated cells, the effect of BUdR was further controlled by probing the blot for myogenin mRNA and quantifying the levels in treated and untreated cells. Steady-state levels of myogenin mRNA in treated L_6 and C2 myoblasts and myotubes were decreased to an average of ~6-9% of the level in untreated cells, respectively (not shown). Molecular sizes are indicated.

Lane 1: C2 myoblasts, day 3; lanes 2, 4: L_6 myoblasts extracted on days 3 and 6, respectively; lanes 3, 5: corresponding L_6 myoblasts treated with BUdR and extracted on days 3 and 6, respectively; lanes 6, 8: C2 myoblasts extracted on days 3 and 7, respectively; lanes 7, 9: parallel cultures of the C2 myoblasts in lane 6, 8 treated with BUdR and extracted on days 3 and 7, respectively; lanes 10-12: adult rat liver, gastrocnemius and heart, respectively.

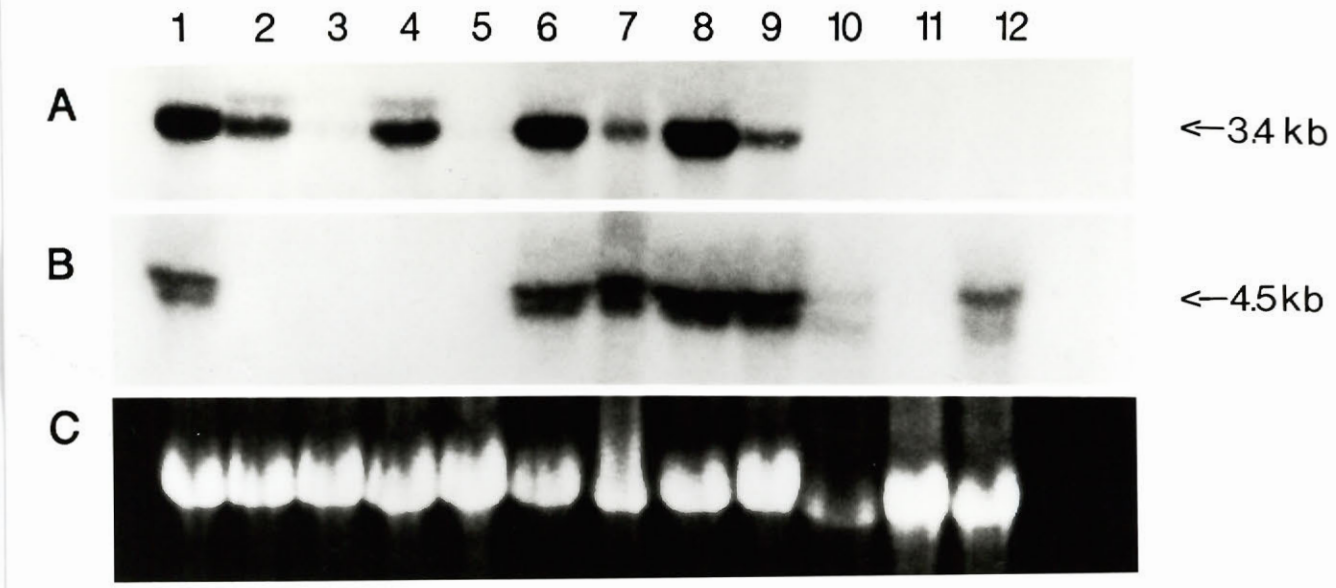
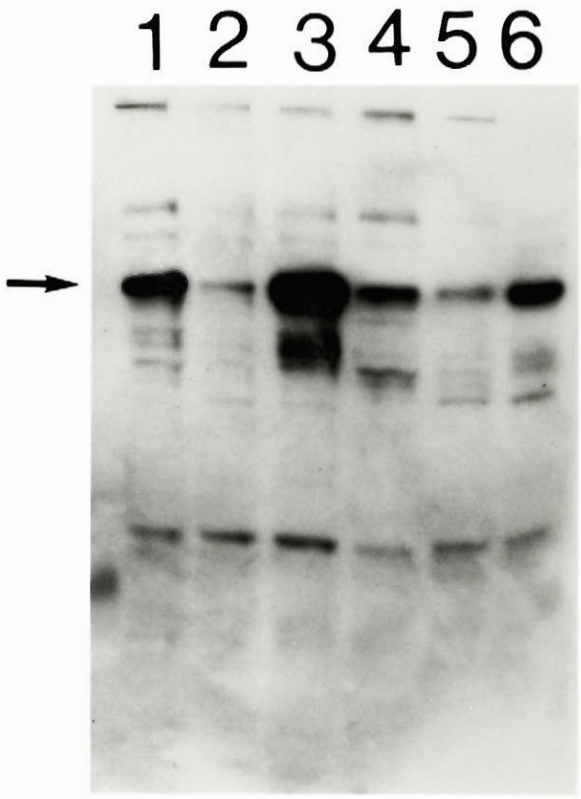

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
←3.4 kb
←4.5kb

Fig. VI:7. BUdR decreases steady-state levels of M-cadherin protein in C2 and L_6 myoblasts

Equal loadings of total cell protein extracts from C2 and L_6 cells were immunoblotted and probed with the sc-69 anti-M-cadherin antiserum. The arrow indicates the position of the species specifically competed by the synthetic 15-mer M-cadherin peptide used to raise the serum (not shown) (M_r 127 kDa).

Lanes 1-3: C2 myoblasts. Lanes 4-6: L_6 myoblasts. Lanes 1,4: Pre-fusion myoblasts. Lanes 2,5: BUdR-treated pre-fusion myoblasts; Lanes 3,6: fused myoblasts.


1 2 3 4 5 6

7.0 DISCUSSION

In this section I will evaluate the significance of the results presented in the previous chapters, and suggest additional experiments which would extend and clarify them. I will first describe the theoretical model which has guided this work.

7.1 Proposed role for cadherin in skeletal myogenesis

For cadherin to play a role in myoblast differentiation, cell contact must be relevant to this process. While many factors influencing myogenesis have been studied, contact between myoblasts as a necessary step leading to their differentiation has not been directly investigated. The only data applicable to this question was published by Emerson (1976). He described experiments which indicated that myoblasts plated in serum-free medium (differentiation-inducing medium) at low cell density undergo a considerable delay in differentiation as compared with cells plated at high density in the same medium. In particular, differentiation of cells plated at low density appeared to occur only after cells had migrated to contact each other, suggesting that cell contact is a determinant of myoblast differentiation.

The significance of myoblast differentiation as being partially controlled by cell adhesion becomes apparent when attempting to understand myogenesis in vivo. During the formation of limb skeletal muscles in vertebrate embryogenesis, presumptive myoblasts migrate from the myotome and invade a presumptive limb bud. Upon reaching a suitable area, presumptive myoblasts stop migrating, begin to differentiate and fuse together to form myotubes. How myoblasts determine when and where to stop migrating and begin differentiating is not known. When cultured in vitro, the

concentration of growth factors such as FGF and TGF-β regulates the differentiation of skeletal myoblasts (see Introduction). Myoblasts gradually deplete these factors from the growth medium and differentiation occurs when a threshold concentration is reached. While this phenomenon is helpful in understanding the regulation of myoblast differentiation in vitro, it is difficult to see how it can apply to the regulation of myoblast differentiation in vivo.

Therefore, in addition to a role for growth factors in the differentiation of skeletal myoblasts, I propose an additional feature of the model describing this process. During organogenesis, presumptive myoblasts migrate into a limb bud where they meet a large number of their counterparts. Specific intercellular adhesive interactions are initiated between these cells. Assuming other necessary conditions are favourable, these interactions result in myoblasts interrupting their migration and initiating the myogenic differentiation program. This leads to plasma membrane fusion and further differentiation. Myoblasts which have failed to interact successfully with other myoblasts may still differentiate eventually, but only as a default outcome, and will do so as mononuclear cells. In this regard, anatomical studies have demonstrated the existence of such differentiated mononuclear muscle cells. For differentiation to be triggered in this way, myoblasts must be able to recognize each other specifically, leading to the postulate of a myoblast-specific adhesion molecule. I propose that a cadherin mediates the myoblast-specific intercellular adhesive role postulated by this model, and that the subtype of this cadherin may be M-cadherin.

The logic of regulating myoblast differentiation through cell contact is clear: Since myoblasts must form a myotube (i.e., a multinucleated cell), it is desirable that

they not differentiate until other myoblasts are nearby. In this way, plasma membrane fusion will accompany biochemical differentiation and muscle cells can be formed, as opposed to differentiated mononuclear muscle cells.

7.2 Is myoblast differentiation regulated by cell density?

The potential role of intercellular adhesion in the control of myogenic differentiation was investigated in experiments where the extent of differentiation was determined for rat L_6 and mouse BC_3H1 (Schubert et al., 1974) myoblasts seeded at either high or low density in serum-free differentiation medium (Appendix 1). Cells seeded at low density did not differentiate efficiently as compared with cells seeded at high density, even after extended periods in culture. Results from these experiments indicate that the concentration of growth factors is not the sole determinant of myogenic differentiation. Furthermore, these results are compatible with the hypothesis that cell density influences myogenic differentiation.

7.3 Immunological studies of cadherin expression in L_6 cells

Using the CADH-1 antiserum, we have shown that blockade of the cadherin expressed in L_6 myoblasts by purified anti-cadherin immunoglobulins can inhibit myoblast fusion (Chapter 5). As the effect of this blockade upon biochemical differentiation was not determined, the antibodies could be inhibiting plasma membrane fusion rather than differentiation per se. However, Mege et al. (1992) have demonstrated that treatment of chicken myoblasts with either monoclonal anti-N-cadherin antibodies or synthetic peptides containing the HAV sequence inhibits both

fusion and biochemical differentiation. My results and those of Mege et al. suggest that intercellular contact as mediated by cadherin is an important control variable of myogenic differentiation.

7.4 Identity of the cadherin expressed in L_6 cells

Another limitation of our immunological studies of cadherin in myogenesis is the lack of identification of the cadherin subtype detected in L_6 myoblasts by CADH-1 antibodies. Reports of the expression of N-cadherin in avian myoblasts suggested that this cadherin might be the subtype expressed in L_6 myoblasts. However, as I have shown here, I could not detect N-cadherin in these cells. While not rigorously proven here, the many common features of M-cadherin and the L_6 cadherin, such as developmental regulation and BUdR-sensitivity, suggest that the L_6 cadherin is M-cadherin. Immunodepletion experiments using the anti-M-cadherin serum have so far yielded inconclusive answers, either because the antibody does not precipitate efficiently or because M-cadherin is labile. Below I propose another approach to investigating the identity of the L_6 cadherin.

7.5 Expression of multiple cadherin subtypes in skeletal myoblasts

Studies in adult and embryonic skeletal muscle have shown that these cells can express multiple cadherins. In particular, chick myoblasts express N-, R- B- and T-cadherin (Hatta et al., 1987; Inuzuka et al., 1991a; Napolitano et al., 1991; Ranscht and Zimmerman, 1991, respectively). In mammals, only N- and M-cadherin are known to be expressed. It is not known whether R-, T- and B-cadherin are expressed in

mammalian myoblasts, or whether an homolog of M-cadherin is expressed in avian myoblasts.

My studies in L_6 myoblasts do not suggest the presence of additional cadherin subtypes in addition to M-cadherin. Although the full spectrum of cadherin expression in these cells can never be proven definitively, evaluating the variety of cadherin subtypes expressed in L_6 or other myoblasts is conceptually simple. An efficient strategy for the surveying of cadherin subtypes is possible using the reverse transcriptase/polymerase chain reaction (RT-PCR). RT-PCR using degenerate or highly conserved primer sequences from short (e.g., ~300 bp), highly conserved cadherin regions have been useful in discovering the protocadherins (Suzuki et al., 1991) and M-cadherin (Donalies et al., 1991). The technique described by these authors can be refined to obtain increased throughput by using automated, fluorescent cycle-sequencing technology rather than traditional sequencing methods. Poly(A)$^+$ RNA isolated from L_6 myoblasts isolated at different time points during their differentiation are reverse transcribed using an oligo-(dT) primer. The resulting cDNAs are then amplified by PCR using several couples of primers derived from short, conserved regions of cadherins. PCR products are then cloned into a suitable sequencing vector and large-scale automated sequencing applied to characterize a statistically significant number of clones. This strategy would permit the simple and economical sequencing of dozens of cDNAs. With the proper controls to ensure that all known cadherins can be amplified using the RT-PCR procedure, a good estimate of the cadherin diversity could be obtained in this way. I have validated this RT-PCR approach by using the primers originally described by Donalies et al. (1991) to clone rat M-cadherin from L_6

myoblasts and to ascertain whether additional cadherins can be isolated from those cells.

The question which arises is, why are several cadherins often co-expressed in a given cell type? This is most puzzling in cells which co-express N- and R-cadherin, whose primary structures are extremely similar and which can mediate heterophilic adhesion with each other. Although the expression of N- and R-cadherin is regulated differently, it is difficult to see how they can mediate specific functions when they are co-expressed and can cross-adhere with each other on apposing cells (Matsunami et al., 1993). This comment can also be made for all group I cadherins (as defined in Pouliot, 1992), which possess the HAV tripeptide and which are very similar to each other. As mentioned above, many HAV-containing cadherins can interact with each other, and as such constitute poor candidates as mediators of myoblast-specific adhesion. This is particularly true of N-cadherin, which is expressed by many cell types during myogenesis, in addition to myoblasts. However, the discovery of group II and III cadherins introduced the notion that cadherins devoid of the HAV tripeptide exist. Particularly interesting in this regard is M-cadherin, which, apart from its lack of the HAV sequence, is very similar to other transmembrane cadherins. Instead of HAV, the putative CAR site region of M-cadherin possesses the sequence FAL. According to amino acid substitution matrices such as BLOSUM62 (Henikoff & Henikoff, 1992), the replacement of histidine by phenylalanine constitutes a highly non-conservative, and thus disfavoured, substitution which can be postulated to confer a high degree of specificity to M-cadherin-mediated interactions. As mentioned previously, it is important to note that M-cadherin has not yet been shown to mediate intercellular

adhesion. In the case of the only other non-HAV-based cadherin, T-cadherin has been shown to mediate calcium-dependent intercellular adhesion in vitro (Vestal & Ranscht, 1992). Unfortunately, it is not known whether T-cadherin can mediate heterophilic adhesion with other cadherin subtypes, particularly group I cadherins. If M-cadherin is shown to mediate calcium-dependent cell adhesion, however, I believe it likely that M-cadherin will show little or no heterophilic adhesion with other cadherins. Furthermore, M-cadherin need not mediate adhesive interactions. Instead, M-cadherin may mediate a myoblast-specific signal transduction role not involving cell adhesion. Transfection experiments similar to those which have been used to determine the adhesive specificity of individual cadherins will be important in answering questions regarding the function of M-cadherin.

Nonetheless, many particularities of M-cadherin support the hypothesis that this cadherin is involved in the regulation of skeletal myoblast differentiation:

1. M-cadherin expression is restricted to embryonic skeletal muscle

So far, M-cadherin is the only cadherin restricted to skeletal muscle, and one of only two cadherins expressed exclusively in the embryo (the other being B-cadherin, Napolitano et al., 1991). Furthermore, I have determined that M-cadherin (contrary to N-cadherin) is expressed in all mammalian myoblast cell lines tested, namely mouse C2 and BC_3H1 cells, as well as rat L_6 and L_8 cells (data not shown), in addition to primary rat and mouse myoblasts.

2. M-cadherin expression is developmentally regulated during skeletal myogenesis

I have shown that the regulation of M-cadherin expression in vitro and in vivo mirrors that of muscle-specific genes, with the exception that M-cadherin is down-

regulated around parturition and is not expressed in the adult. Furthermore, Moore and Walsh (1993) have shown that M-cadherin is re-induced upon the experimental induction of regeneration of adult muscle, but not by denervation. This is in contrast to N-cadherin, which is induced upon both experimental denervation and regeneration in a fashion similar to N-CAM (Hahn and Covault, 1992).

3. M-cadherin expression is susceptible to BUdR

I have shown that BUdR treatment of myoblasts is a potent inhibitor of M-cadherin expression, but has no effect upon N-cadherin mRNA levels (Chapter 6). M-cadherin is the only cadherin for which this effect has been reported. So far, genes which are directly or indirectly susceptible to BUdR belong either to the HLH family or to the group of muscle-specific genes. Substitution of BUdR in lieu of thymidine nucleotides in the promoter of HLH genes is believed to reduce the affinity of HLH proteins to these promoters, thereby inhibiting their transcription. In the case of muscle-specific genes, many such genes are characterized by the presence of promoter elements to which HLH proteins such as MyoD1 or myogenin bind to activate transcription (cf. Lassar et al., 1989). Therefore, in the case of muscle-specific genes the inhibition of transcription by BUdR results from a decrease in the concentration of HLH proteins, as opposed to a direct effect of BUdR substitution upon these genes. This mechanism is the likeliest explanation for the inhibition of M-cadherin expression by BUdR, and suggests M-cadherin is under the control of the program of myogenic terminal differentiation. Nonetheless, the possibility exists that the promoter of the M-cadherin gene itself contains putative BUdR-sensitive elements. So far, no such elements have been found in the only two cadherin promoters which have been

studied, those of P- and E-cadherin (Faraldo & Cano, 1993; Behrens et al., 1991, and Ringwald et al., 1991, respectively). Tapscott et al. (1989) have used a transfection strategy to show the direct involvement of MyoD1 in myogenic regulation. In that experiment, a MyoD1 expression vector was transfected into BUdR-treated fibroblasts to demonstrate that exogenously-supplied MyoD1 was sufficient to re-induce expression of muscle-specific genes, and that inhibition of these genes did not result from the presence of BUdR-sensitive elements in their promoters. Similarly, we will use this strategy to determine if M-cadherin is directly under the control of MyoD1 by transfecting a MyoD1 expression vector into BUdR-treated L_6 myoblasts, and comparing levels of M-cadherin mRNA with those of cells transfected with an antisense MyoD1 vector. This will provide direct evidence that M-cadherin can be regulated by an HLH protein and that its promoter is not susceptible to BUdR substitution.

4. The primary structure of the M-cadherin EC1 domain is unique

M-cadherin has long evolved independently from other cadherins (Chapter 2) and is distinct from them. Furthermore, the region corresponding to the CAR site of M-cadherin encodes an FAL tripeptide, not HAV. This substitution should confer a unique tertiary structure for the putative CAR site of M-cadherin, and suggests that putative interactions mediated by M-cadherin are likely to be much more specific than those mediated by group I cadherins.

The above support the hypothesis that M-cadherin is involved in terminal myoblast differentiation. Nevertheless, little direct evidence exists linking M-cadherin to myoblast differentiation. Several types of experiments can be envisioned to address

this question, in which the goal is either to perturb or enhance M-cadherin function:

1. treatment of L_6 myoblasts with antisense oligonucleotides

I have generated preliminary data which indicate that co-culture of L_6 myoblasts with an antisense oligonucleotide inhibits their fusion in conditions where the control oligonucleotide has little effect. However, I have not ascertained the effect of this treatment upon differentiation per se. While relatively easy to implement, this strategy is not very flexible. In particular, experiments with several oligonucleotides encoding different sequences must be performed, and effects upon mRNA and protein levels must be demonstrated. Other approaches now appear preferable (see below).

2. transfection experiments

Our laboratory has generated several permanent lines of transfected L_6 cells which express nearly full-length sense or antisense M-cadherin constructs. Cells transfected with the antisense construct show a moderate decrease in levels of M-cadherin transcripts, whereas no effect is observed in cells transfected with the sense construct. We are now characterizing the extent of fusion and differentiation in these lines. An important limitation of this approach is the difficulty of obtaining cell lines with levels of expression by the construct which are sufficiently high to hybridize with enough M-cadherin transcripts to impart physiological effects. One solution is to evaluate the effect of the constructs in transient, rather than permanent, transfectants. However, this requires high transfection efficiency. Fortunately, preliminary experiments using the LipoAMINE transfection procedure (Gibco-BRL) indicate that efficient transfection (~20%) can be obtained in L_6 cells. Optimization of the transfection protocol may increase this proportion.

3. co-culture of L_6 cells unto M-cadherin-expressing cells

The effect of M-cadherin upon differentiation and fusion can be assessed directly by seeding L_6 myoblasts on a monolayer of L cells (which do not express cadherins) transfected with an expression vector encoding M-cadherin. If M-cadherin is regulated during myogenesis, enhanced differentiation and fusion should follow, as measured by quicker induction of myogenin and muscle-specific genes and more extensive fusion. Controls would include transfection of N-cadherin instead of M-cadherin, as well as transfection of a construct expressing a protein both unrelated to the cadherins and inactive in myogenesis.

4. co-culture of L_6 myoblasts with FAL-containing synthetic peptides

The role of M-cadherin during myogenesis in vitro can also be investigated directly using the strategy which led to the identification of the cadherin CAR site. The effect of synthetic peptides encoding the FAL region of M-cadherin upon myoblast fusion and differentiation could be contrasted with those of peptides encoding the CAR region of HAV-based cadherins. A non-sense FAL peptide would also be used to control directly for effects of FAL peptide unrelated to the native sequence. In L_6 myoblasts, which do not express N-cadherin and which may not express other HAV-based cadherins, the HAV peptide should have little effect, whereas the FAL peptide should be a potent inhibitor of myogenesis if indeed M-cadherin plays an important role in myogenesis. In C2 myoblasts, which express both N- and M-cadherin, treatment with HAV peptides might demonstrate a possible permissive role of N-cadherin in addition to effects mediated by M-cadherin. Initial experiments using a 10-mer FAL synthetic peptide indicate that the pI of this region is near 7, making it insoluble in the

physiologically-relevant pH range. Peptides encoding other sequences will be evaluated.

7.6 Possible role of N-cadherin

As with other tissues, skeletal myoblasts are known to co-express multiple cadherins. In mammalian myoblasts, both N-cadherin and M-cadherin are expressed. The data I have presented here suggest that N-cadherin is not essential for myogenesis in vitro, as L_6 myoblasts differentiate and fuse even though N-cadherin transcripts are not detectable. This is reminiscent of the loss of MyoD1 expression in L_6 myoblasts (Wright et al., 1989). Rather, L_6 myoblasts principally express myogenin (another member of the HLH family), and the partial functional redundancy shared by members of the family appears to be sufficient to account for the needs of myogenesis in vitro.

Several explanations for the apparent role of N-cadherin in avian myogenesis (Mege et al., 1992; see also Chapter 6) can be put forth. Firstly, N-cadherin may play an indirect, facilitatory role in myoblast fusion. Cadherins have been proposed to exhibit cooperative behaviour via their cytoplasmic domain (Kemler & Ozawa, 1989), such that interfering directly with one cadherin subtype might indirectly disturb the interactions of another subtype involved in myoblast-specific adhesion. Secondly, steps subsequent to the formation of myotubes were not investigated here, most notably the innervation which follows myotube formation. In this respect, Hahn and Covault (1992) have shown that the pattern of expression of N-cadherin during skeletal myogenesis is consistent with neuronal regulation. N-cadherin protein and mRNA levels plummet when muscles become innervated during embryogenesis, and agents

which inhibit the transmission of nerve impulses re-induce expression of N-cadherin. Furthermore, N-cadherin can be re-induced upon experimental denervation. Lastly, the rate of contraction appears to regulate the levels of N-cadherin expression. These data are in agreement with results described by Knudsen et al., (1990c), who found little changes in N-cadherin levels during the differentiation of chick skeletal myoblasts in culture. However, MacCalman et al. (1992) found that levels of N-cadherin mRNA decreased as C2 myoblast differentiate aneurally. This decrease may be due to regulation of the N-cadherin gene by growth factors rather than by the program of terminal differentiation per se. Such a situation is observed in the regulation of the $\alpha_5\beta_1$ integrin in cultures of differentiating human myoblasts. This integrin is down-regulated by growth factor concentration instead of terminal differentiation (Blaschuk & Holland, submitted). Furthermore, MacCalman et al. did not assess the effect of treatment BUdR, as I have done in Chapter 6. The lack of inhibition of N-cadherin transcription by BUdR suggests that N-cadherin expression is not regulated by the program of terminal differentiation. Assuming that specific developmental regulation implies specific function, N-cadherin would thus appear to be involved in later stages of myogenesis, such as innervation, rather than in functions necessitated during terminal differentiation.

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9.0 Appendix A: Effect of cell density upon myoblast differentiation

The effect of cell density upon terminal myoblast differentiation was evaluated by measuring the activity of creatine kinase in low and high density cultures of myoblasts. Rat L_6 or mouse BC_3H1 myoblasts cultured in growth medium (DMEM +10% FBS; DMEM + 20% FBS, respectively) were trypsinized and washed three times in PBS. Two cell densities were used to seed 90 mm bacterial culture dishes containing 10 ml of differentiation medium: 7.65 X 10^4 cells (low density) and 7.65 X 10^5 cells per dish (high density). These seeding densities correspond to 1000 and 10 000 cells per mm^2, respectively. Cells were seeded in serum-free DMEM supplemented with ITS as per manufacturer's instructions (Gibco-BRL) to induce differentiation. The use of bacterial dishes and serum-free medium prevented attachment of cells, such that they remained in suspension. At the low seeding density, cells remained essentially single. After 48 and 96 hours in culture, cells were pelleted in ice-cold PBS, washed twice and homogenized on ice in PBS + 2 mM DTT with 50 strokes of a Dounce homogenizer. The suspension was microfuged briefly and the supernatant removed. The protein concentration of an aliquot of the supernatant was determined using the BCA method as per manufacturer's instructions (Pierce). Creatine kinase activity was measured spectroscopically for the remainder of the supernatant using the Boehringer Mannheim CK-NAC kit, as per manufacturer's instructions. Values are expressed in mIU per mg of supernatant protein, followed by the range for two experiments. The creatine kinase activity of undifferentiated L_6 and BC_3H1 myoblasts was determined to be 20 and 10 mIU per mg of protein, respectively.

Myoblast	L_6		BC_3H1	
Cell density	Low	High	Low	High
Time post-seeding				
+48 hours	180 (21)	750 (81)	90 (82)	395 (43)
+96 hours	205 (27)	1100 (74)	223 (25)	993 (96)