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Lee, Chung-Hyun,

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Functional Study of C-cadherin in Early Xenopus Development

by

Chung-Hyun Lee

DISSERTATION

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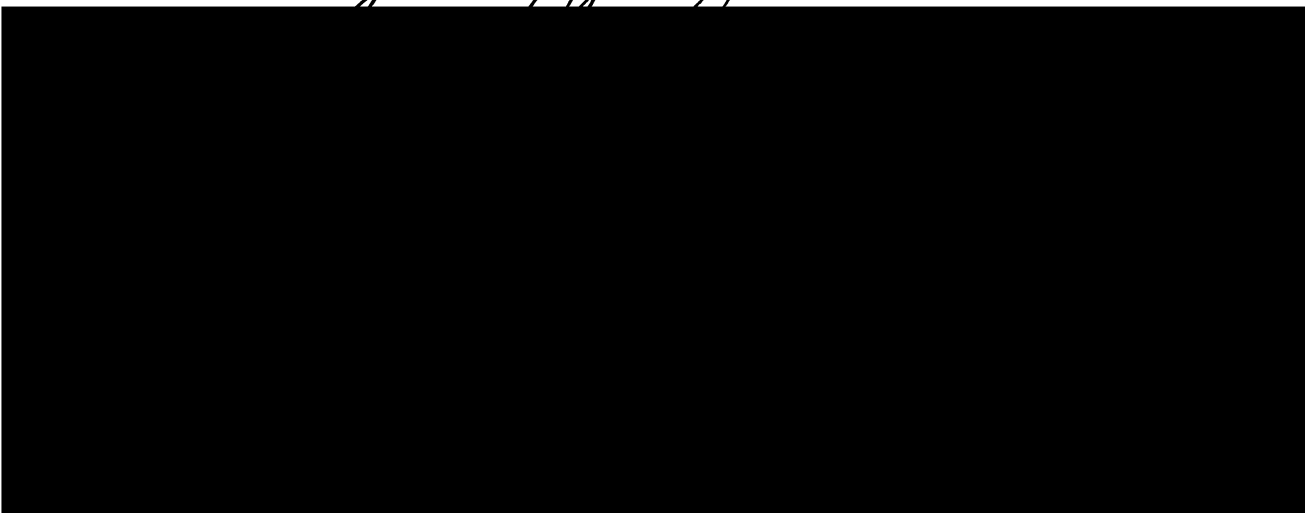
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FUNCTIONAL STUDY OF C-CADHERIN IN EARLY *XENOPUS* DEVELOPMENT

Chung-Hyun Lee

Dr. Barry M. Gumbiner, Ph.D.
Chairman, thesis committee

DISSERTATION ABSTRACT

Calcium-dependent adhesion was observed in early cleaving *Xenopus* embryos and cadherins were postulated to be involved. C-cadherin was first identified in *Xenopus* oocytes, eggs and cleaving embryos using a pan-cadherin antibody. In order to generate a specific tool for C-cadherin, C-cadherin was cloned and sequenced.

To study the function of C-cadherin during early cleavage stages, an antisense approach was used to deplete the maternal pool of C-cadherin mRNA. Antisense oligodeoxynucleotides (oligos) were injected into immature oocytes and fertilizable eggs were generated using the oocyte transfer technique. One modified oligo (m97) was able to deplete the C-cadherin transcripts; after maturation, little or no C-cadherin was detected. A mutant construct, with deletion of

the oligo hybridization site, was generated to do rescue experiments. When injected into two-cell stage embryos, the mutant construct, like the wild-type construct, was expressed at high levels, caused surface lesions at the gastrula stage, and the mutant protein was recognized by C-cadherin antibodies, suggesting that it was functional. Out of over 400 oocytes used in the experiment, only 6 fertilized and developed to the tailbud stage. Although none of the embryos showed obvious adhesion defects, the results are inconclusive due to the low sample size.

The function of C-cadherin was also studied in the cells of the dorsal involuting marginal zone (DIMZ), since they express C-cadherin and rely heavily on cell-cell contact for movements. A deletion mutant consisting of the extracellular and transmembrane domains (Ctrunc) was constructed to act as a dominant negative inhibitor. Injection of Ctrunc mRNA into the prospective DIMZ caused a reproducible defect in gastrulation movements. The defect, an inability to complete involution and to close the blastopore, was cadherin specific, since it could be rescued by expressing full-length C-cadherin. Ctrunc seems to inhibit morphogenetic movements directly, because it specifically blocked the activin-induced elongation of animal cap explants from injected embryos. These results demonstrate that C-cadherin plays a critical role in *Xenopus laevis* gastrulation movements, and they support the hypothesis that cadherins can mediate cellular rearrangements during tissue morphogenesis.

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Abbreviations

A	Anterior
An	Animal hemisphere
BC	Blastocoel cavity
b.p.	Base pair
BL	Blastopore lip
CT	Cytoplasmic tail
Ctail	C-cadherin mutant (tail domain)
Ctrunc	C-cadherin mutant (extracellular and transmembrane domain)
D	Dorsal
dATP	Deoxyadenosine triphosphate
DEPC	Diethyl pyrocarbonate
DIMZ	Dorsal involuting marginal zone
DMZ	Dorsal marginal zone
DNA	Deoxyribonucleic acid
EC	Extracellular
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacidic acid
EF1α	Elongation factor 1α
Etrunc	E-cadherin mutant (extracellular and transmembrane domain)
HCG	Human chorionic gonadotropin
IMZ	Involuting marginal zone
Kb	Kilobase
kD	Kilodalton
MBT	Midblastula transition
MDCK	Mardin-Darby canine kidney
MMR	Modified Barth's solution with Hepes buffer
mRNA	Messenger ribonucleic acid
mut B	C-cadherin mutant (with deletion of nucleic acids 430-445 recognized by oligo m97)
NaCl	Sodium chloride
ng	Nanogram
NIMZ	Noninvoluting marginal zone
NP-40	Nonidet P-40
Ntrunc	N-cadherin mutant (extracellular and transmembrane domain)

oligos	oligodeoxynuleotides
PBS	Phosphate buffered saline
PIPES	Piperazine-N'-N'-bis[2-ethane-sulfonic acid]
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
SSC	Sodium chloride, sodium citrate
STV	Sterile trypsin versene
TM	Transmembrane
UTP	Uridine triphosphate
V	Ventral
Vg	Vegetal
Xgal	5-bromo-4-chlor-3-idolyl-galactopyranosid
YP	Yolk plug

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

I. Adhesion molecules:

At a basic level, cell adhesion molecules act as glues to hold cells together to form tissues, and to hold tissues together to form the organism. However, cell adhesion has been shown to play a more dynamic role during development, since changes in adhesivity can affect many aspects of morphogenesis, including movement, morphology, growth, and differentiation. They act not only to organize the embryos structurally, but they can also influence intracellular signaling events. Understanding the roles of adhesion molecules is therefore important in analyzing developmental processes. The major families of adhesion molecules studied in development are members of the Ig superfamily, the integrins, and the cadherins.

Members of the Ig superfamily bind in a homophilic and Ca^{2+}-independent manner. One well known member is N-CAM, which has immunoglobulin-like structures in the extracellular domain and a variable transmembrane and cytoplasmic domain, generated by alternate splicing (Cunningham et al, 1987; Williams and Barclay, 1988). N-CAM is particularly known for its role during the development of the central nervous system.

Integrins, best characterized by the fibronectin receptor, are composed of two subunits (α and β) and interact primarily with extracellular matrix (ECM) proteins, such as fibronectin and laminin (Hynes, 1992). They function as transmembrane linkers to mediate interaction between the extracellular matrix and the actin

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cytoskeleton. They often interact with matrix proteins in a domain which includes the amino acids RGD, and the addition of the tripeptide alone can disrupt many integrin mediated adhesion.

The last major family of adhesion molecules in development are the cadherins, which is discussed in more detail below.

II. Cadherins.

Cadherins belong to a family of Ca^{2+}-dependent, homotypic cell-cell adhesion molecules (Geiger and Ayalon, 1992; Takeichi, 1990). Structurally, they have a single transmembrane domain and a semiconserved extracellular domain containing 4 cadherin repeats for specific recognition. There is evidence suggesting that the amino acid sequence HAV, which is located in the first cadherin repeat of most cadherins, is the cadherin recognition sequence, much like the amino acids RGD located in many ECM proteins for binding to integrins (Hynes, 1992). The strongest evidence suggesting a role as a cadherin recognition sequence is the inhibitory effect of synthetic HAV-containing peptides on cadherin mediated adhesion (Blaschuk et al., 1990). The amino acids flanking the HAV sequence were also shown to play an important role, possibly providing specificity between different cadherins (Takeichi, 1990). In addition, cadherins, with the exception of T-cadherin, share a highly homologous cytoplasmic domain, which is believed to interact directly with catenins and to link them to the actin cytoskeleton.

Catenins were initially discovered as cadherin associating proteins in co-immunoprecipitation experiments. The three members, known as α, β, and γ catenins, have been cloned. α-catenin

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has homology to vinculin and β and γ-catenins have homology to *Drosophila* armadillo and human plakoglobin, respectively (Gumbiner and McCrea, 1993). Cadherins must interact with catenins and the actin cytoskeleton for stable adhesion, as suggested by deletion studies (Ozawa et al., 1990; Takeichi, 1991). Takeichi and his group also discovered a lung carcinoma PC9 cell line, expressing high levels of both E-cadherin and β-catenin, but with reduced cadherin-mediated aggregation activity (Hirano et al., 1992). After transfection with neural α-catenin, however, the cells displayed tight adhesion and appeared epithelialized, further suggesting that interaction with catenins was essential for stable adhesion. β-catenin, which most tightly associates with the cadherins, has been extensively studied recently for its possible role as a signaling molecule (McCrea et al., 1993). Ectopic expression of β-catenin into *Xenopus* embryos leads to duplication of embryonic axis, much like that observed with Wnt-1, which is thought to be a developmental signaling molecule (McMahon and Moon, 1989). The mechanism by which β-catenin is linked to the signaling pathway is not known.

The three major types of cadherins were originally delineated as E-, N-, and P-cadherins, representing epithelial, neural, and placental cadherins, respectively; and new cadherins were typed according to homology to established cadherins and their location of discovery. Several new members, however, have been identified which do not fit into the delineation, such as M-cadherin, B-cadherin, and T-cadherin (Geiger and Ayalon, 1992).

Although cadherins adhere primarily in a homotypic manner between adjacent cells, some heterotypic binding has been observed

between different members of the cadherin family (Nose et al., 1990; Volk et al., 1987). It is also possible that lateral interactions exist between cadherins on the same cell (Kintner, 1992). This may, for example, provide a mechanism by which cadherins cluster at adherens junctions to increase their structural strength.

The role of cadherins in the formation and organization of adherens junctions has been extensively studied in many epithelial cell lines. In MDCK cells, for example, E-cadherin is localized to adherens junctions, which form an adhesion belt around each cell (Gumbiner et al., 1988). The formation of adherens junctions is essential for the formation of tight junctions, which separate the basolateral and apical sides of the epithelium. In low Ca^{2+} media, adherens junctions will disband followed by tight junctions, presumably due to disruption of cadherin-mediated adhesion. Adherens and tight junctions will reform if calcium alone is returned to the media, but not if antibodies against E-cadherin is also present. E-cadherin mediated adhesion, therefore, appears to be essential for the formation and maintenance of junctional complexes in MDCK cells (Gumbiner et al., 1988).

Since its initial discovery, the cadherin family of adhesion molecules has expanded greatly to include proteins which are structurally different from the "classical" cadherins. Desmogleins and desmocollins, components of desmosomes, belong to the cadherin superfamily of Ca^{2+}-dependent adhesion molecules, with a high degree of sequence homology to the classical cadherins in both the extracellular and transmembrane domains (Koch et al., 1992). There is, however, little homology in the cytoplasmic tail,

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consistent with their cytoskeletal link to intermediate filaments rather than the actin cytoskeleton. A cadherin-like protein has also been cloned from *Drosophila*, but very little is known about its function (Oda et al., 1994).

III. Cadherins and development.

In vivo and in vitro studies suggest that cadherins are not only involved in the structural organization of cells but also in regulation of morphogenic processes. A strong correlation has been observed between the expression of different cadherin-types during development and morphogenic events.

For example, cells transfected with different members of the cadherin family will sort out from each other and adhere preferentially with their own type (Takeichi, 1988). In vivo, one example of where a similar process might occur is in the formation of the neural tube. Cells from the ectoderm destined to separate and form the neural tube will start expressing N-cadherin, while the remaining ectoderm continues to express E-cadherin (Detrick et al., 1990). This suggests that expression of a different cadherin may be involved in the separation of tissues.

In another example, E-cadherin appears to play an important role in mouse compaction, which leads to the polarized organization of mouse embryos (Fleming and Johnson, 1988). Compaction induces the loosely associated blastomeres to form a tight transporting epithelium, concomitant with the redistribution of the initially diffusely distributed E-cadherin into cell-cell contact sites (Vestweber et al., 1987). Antibodies against E-cadherin can

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temporarily delay compaction, suggesting that E-cadherin is critical for the change in organization (Fleming and Johnson, 1988). Since there is little change in the level of E-cadherin during compaction, however, expression alone is not sufficient. In addition, exposure to phorbal esters can induce premature compaction, suggesting that cadherins may be regulated by intracellular signaling (Fleming and Johnson, 1988).

Finally, mesenchymal to epithelial transition is accompanied by alteration in cadherin expression, suggesting that different cadherins are more suited for different types of cells (Ekblom, 1989). How much the change in cadherin expression contribute to the change in cell morphology is not clear.

IV. Cell adhesion, cell migration, and development:

Cell adhesion and cell migration are interdependent processes. The degree of adhesion will not only determine whether cells can bind to the substrate, but also whether it is permissive for motility (DiMilla et al., 1993). Very weak adhesion will prevent binding and traction between the cell and the substrate, so that cells are not able to attach and move. Very strong adhesion with the substrate will immobilize the cells and also prevent motility. The level of adhesion, therefore, must not only be optimal but also dynamic since cells need to attach and detach as it moves across a substrate (Schmidt et al., 1993).

The role of adhesion molecules and cell migration in development has been extensively studied with members of the integrin family

(Smith et al., 1990). During embryogenesis, cells rearrange extensively in order to organize the body plan and motility plays a critical role. In *Pleurodeles*, for example, RGD peptides or antibodies against the fibronectin receptor can block gastrulation by preventing mesodermal cells from migrating across the fibronectin-coated blastocoel roof, which is required for the formation of the anteroposterior axis (Smith et al., 1990). In *Xenopus laevis* , however, RGD peptides do not block gastrulation since the mesodermal cells do not rely on migration for movement. Since *Xenopus* embryos rely more on cell-cell interactions, another class of adhesion molecule, namely cadherins, may provide the traction for movement.

V. *Xenopus laevis* development.

Xenopus provides a manipulable and well described in vivo system to study cadherin functions. Not only have the major cadherins been identified, the developmental biology has also been studied in detail (Keller and Winklbauer, 1992; Levine et al., 1994; Muller et al., 1994; Newport and Kirschner, 1982) (figure 1).

Most proteins required until the midblastula transition (MBT), when zygotic transcription is initiated, are translated during oocyte maturation from maternal transcripts and stored within vesicles. After fertilization, the *Xenopus* embryo undergoes 12 synchronized and rapid cleavages before reaching the MBT (Newport and Kirschner, 1982). The stored vesicles fuse to the cleavage furrow to supply both lipid and protein materials for the formation of the new membranes (see figure 2). Since the embryos rely solely on the

maternally derived material until zygotic transcription is initiated, manipulations to the maternal transcripts in the oocyte cannot be compensated by the embryo until the MBT. By the blastula stage, the embryo is made up of several thousands of cells, surrounding a fluid-filled cavity. Ca^{2+}-dependent adhesion between the newly developed membranes of the cleavage furrow suggests that the blastomeres are held together by a member of the cadherin family. The outer layer of cells form a tightly sealed epithelium, which successfully separates the internal environment from the external medium.

Gastrulation transforms the blastula, a ball of undifferentiated cells, into a well organized elongated tadpole with distinct body axes (figure 3). By the end of gastrulation, the animal/vegetal, dorsal/ventral, and the anterior/posterior axes are established (Keller and Winklbauer, 1992). Most of the rearrangements occur in the dorsal marginal zone, which becomes induced by growth factors to greatly increase its surface area by radial and mediolateral cell intercalation. The former involves thinning of the cell layers (interdigitation) to create a larger surface area and the latter involves rearrangement of cells to form a longer and narrower population (convergence and extension). Much of the increase in surface area is internalized, where the involuted cells move and extend across the blastocoel roof by convergence and extension, and leads to the organization of the anteroposterior body axis.

This movement by convergence and extension appears to be independent of fibronectin, located primarily on the blastocoel roof. Keller and his colleagues found that involution and closure of the

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blastopore lip can occur in the absence of a blastocoel roof (Keller and Jansa, 1992). They also discovered that explanted involuting marginal zone (IMZ) cells do not converge and extend until IMZ cells in control sibling embryos start involution. Therefore, the force producing these movements must be generated by cells within the IMZ and regulation must be intrinsic to the explant.

VI. Cadherins in *Xenopus*.

A 120 kD member of the cadherin family, C-cadherin, was identified in early cleaving stage *Xenopus* embryos using a pan-cadherin antibody (Choi et al, 1990). We have since cloned C-cadherin and specific antibodies have been generated (Levine et al., 1994; Lee and Gumbiner, 1994). It is first expressed at high levels during meiotic maturation and stored within vesicles (figure 1). During cleavage, these vesicles are delivered to the cleavage furrow, where they are thought to provide the adhesion between the blastomeres. Two other cadherins have been identified during the early cleavage stages but they appear to be similar or identical to C-cadherin. EP-cadherin has been cloned and sequence comparison reveals differences in only 4 amino acids, suggesting that they are probably 2 alleles of the same gene (Ginsberg et al., 1991; Levine et al., 1994). Like C-cadherin, immunofluorescence studies suggest that it is distributed throughout the early embryo. U-cadherin/XB-cadherin, representing a minor maternal cadherin pool, has similar molecular weight and nucleotide sequence (90%) to C-cadherin but it is unclear whether they are derived from different alleles or from different genes (Muller et al., 1994). Although they have similar

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distribution patterns early in development, they diverge later, suggesting that they might be encoded by a distinct genetic loci. After the midblastula transition, the embryo starts expressing its own C-cadherin, along with E-cadherin, whose expression is initially localized to the most outer epithelial layer of cells. N-cadherin is not expressed until neurulation (Detrick et al., 1990). In the early cleaving stages, the cells expressing C-cadherin are static, dividing quickly but undergoing little cell rearrangements. During gastrulation, however, C-cadherin may have a more active role.

During convergence and extension, the dorsal IMZ cells extend large lamellar protrusions directly on neighboring cell surfaces and exert traction to pull and intercalate between one another to form the longer and narrower shape (Keller et al., 1992). This cell to cell contact is critical for cell rearrangements, since migration on fibronectin plays only a minor role. In addition, very little ECM proteins are found on the IMZ cells. The only other major adhesion molecules known to be expressed at this stage of development are the cadherins, particularly C-cadherin, suggesting that perhaps it provides the traction between the intercalating cells. Therefore, interruption of cadherin-mediated adhesion in the IMZ is expected to disrupt normal gastrulation movements.

Figure 1.1. Cadherin expression in early *Xenopus* Development. Most of the C-cadherin required before the MBT is translated during meiotic maturation and stored within vesicles. E-cadherin and N-cadherin are expressed during gastrulation and neurulation,

Oogenesis

oocyte 30 μm

Full-grown oocyte

Meiotic Maturation 6 hrs

Unfertilized egg

C-cadherin accumulation

Unfertilized egg

Fertilization

2-cell stage

Rapid synchronous cleavages

4000-cell midblastula

C-cadherin recruitment to cell surface

4000-cell midblastula

Midblastula transition

early gastrula

Gastrulation neurulation

late neurula

E-cadherin expression, in ectoderm

N-cadherin expression (C. Kintner)

Figure 1.2. Formation of the new cleavage furrow. Vesicles containing C-cadherin and other protein/lipid components required for the formation of the new membranes are formed during meiotic maturation. They fuse to the cleavage furrow to form new membranes. By the midblastula transition, the embryo is made up of several thousands of cells, held together by calcium-dependent adhesion proteins. Tight junctions are also present in the outer layer of cells and successfully separates the internal environment from the external medium.

blastocoel cavity

tight junctions
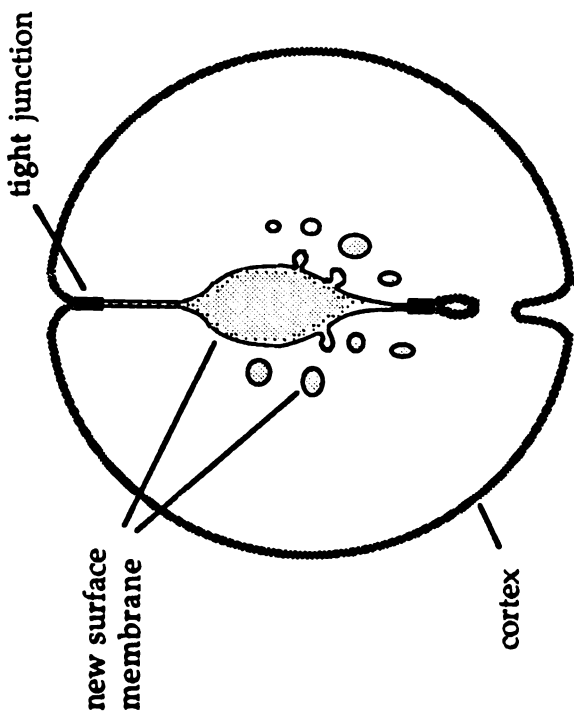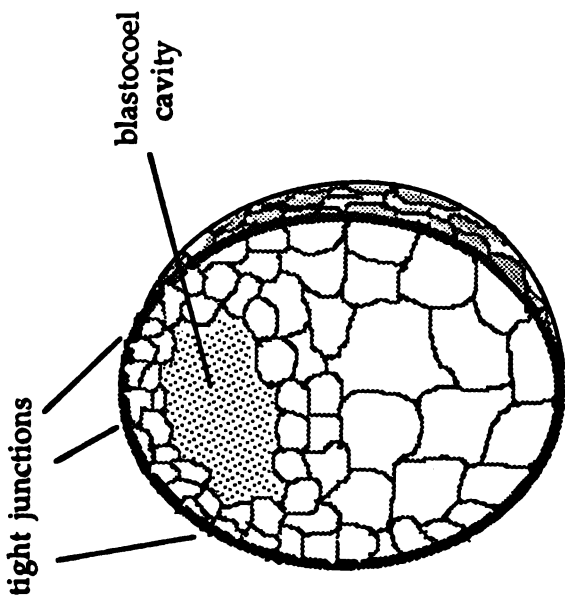
hemisected late blastula

tight junction

new surface membrane

cortex

egg cleavage furrow

Figure 1.3. *Xenopus* gastrulation. A. Late blastula stage embryo. B. Early gastrula stage embryo. C. Late gastrula stage embryo. D. Tailbud stage embryo. **A**, anterior. **An**, animal hemisphere. **BL**, blastopore lip. **BC**, blastocoel cavity. **D**, dorsal. **IMZ**, involuting marginal zone. **NIMZ**, noninvoluting marginal zone. **P**, posterior. **V**, ventral. **Vg**, vegetal hemisphere. **YP**, yolk plug.
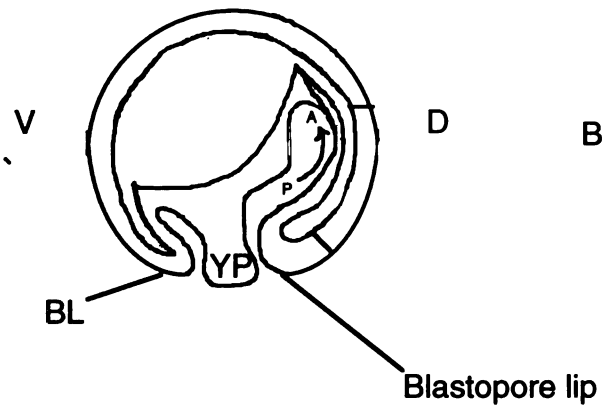
An

BC

NIMZ

IMZ

Vg

A. Late blastula stage embryo

V

D

Blastopore lip

B. Early gastrula stage embryo

V

D

A

P

YP

BL

Blastopore lip

C. Late gastrula stage embryo

Neural tube

Notochord

Brain

Cement gland

D. Tailbud stage embryo

CHAPTER 2
CLONING AND SEQUENCING
C-CADHERIN

CHAPTER 2: CLONING AND SEQUENCING *XENOPUS* C-CADHERIN.

I. Abstract.

A 120 kD member of the cadherin family, called C-cadherin, was identified in *Xenopus* oocyte, egg, and early cleaving embryos using a pan-cadherin antibody (Choi et al., 1990). Since the 120 kD protein was smaller in size than the 140 kD E-cadherin and was not recognized by the anti-E-cadherin antibodies available, C-cadherin was likely to be a novel member of the cadherin family. Since we did not have any method by which to distinguish C-cadherin from other members of the cadherin family or a tool to do functional studies, C-cadherin was cloned and specific antibodies were generated (Levine et al., 1994; Lee and Gumbiner, 1994).

The last 250 b.p. from the cytoplasmic domain of *Xenopus* N-cadherin was used as a probe to clone C-cadherin from a *Xenopus* oocyte cDNA library (Detrick et al., 1990). We used the oocyte library because it is enriched for maternal cadherin cDNAs. Southern blotting suggested that all 19 positive clones isolated from the library were similar or identical. Sequence comparison revealed that C-cadherin had a high degree of homology with other members of the cadherin family, particularly to mouse E-cadherin (56%) and P-cadherin (60%) (Levine et al., 1994). Specific antibodies were generated and western blotting confirmed that C-cadherin was a novel member of the cadherin family.

II. Introduction:

Early developmental biologists recognized the presence of a Ca^{2+}-dependent intercellular adhesion system in amphibian embryonic cells. In the early *Xenopus* embryo, the blastomeres are easily dissociated in Ca^{2+}-free conditions, a phenotype very characteristic of cadherin mediated adhesion, without disturbing the cell cycle or cytokinesis (Nomura et al., 1986; Nomura et al., 1988). Since E-cadherin is known to mediate adhesion in early mouse and chick embryos, it was expected that E-cadherin would mediate the adhesion between early *Xenopus* blastomeres (Fleming and Johnson, 1988).

Xenopus E-cadherin was first identified in A6 cells, a *Xenopus* cell line, by cross-reactivity to an antibody raised against canine E-cadherin (Choi and Gumbiner, 1989). It has since been purified and specific antibodies have been generated (Choi and Gumbiner, 1089). Interestingly, it was not expressed at detectable levels in western blots until gastrulation, suggesting that another member of the cadherin family was mediating the adhesion at the earlier stages.

Using a pan-cadherin antibody raised against a peptide corresponding to a highly conserved region from the cytoplasmic domain of *Xenopus* N-cadherin, a 120 kD protein called C-cadherin was identified in the *Xenopus* oocyte, egg, and early cleaving embryo (Choi et al, 1990). However, this pan-cadherin antibody recognized all cadherins tested and could not be used to study C-cadherin specifically.

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To generate a C-cadherin-specific tool by which to do functional studies, the C-cadherin cDNA was cloned from an *Xenopus* oocyte cDNA library. Sequence comparison and western blotting using antibodies generated against the extracellular domain of C-cadherin expressed in E. coli. confirmed that it was a novel member of the cadherin family and that the antibodies were C-cadherin specific.

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III. Results:

A. Generation of a cadherin probe and screening of a *Xenopus* oocyte cDNA library.

The cytoplasmic domain, with the exception of T-cadherin, is highly conserved among members of the cadherin family (Takeichi, 1990). To isolate the C-cadherin cDNA, the last 250 b.p. from the *Xenopus* N-cadherin cytoplasmic domain, was used as a probe (kindly provided by C. Kintner; Detrick et al., 1990). *Xenopus* N-cadherin/pSP72 was digested with Pvu II and Hind III, the 250 b.p. DNA fragment was purified on a 5 % acrylamide gel, and ^{32}P-dATP labeled using a random primer kit. This probe was used to screen 250,000 plaques from a λgt10 *Xenopus* oocyte cDNA library (kindly provided by D. Kimmelman) and 19 positive plaques were isolated and purified (Sambrook et al., 1989).

The inserts were isolated from the positive clones to determine the size of the cDNA inserts (data not shown). The largest clone ("7B3"), a 3.5 Kb long insert, was purified and subcloned into a bluescript SK+ vector, which permits more manipulations to the clone.

B. Southern blotting to determine the relative degree of homology between the 19 clones isolated.

To determine whether the 19 positive clones isolated from the λgt10 library were clones encoding the same or highly related cadherins, Southern blots were performed using the largest insert (7B3) as a probe under low and high stringency conditions (Sambrook et al., 1989).

Inserts from the 19 positive clones, including the 7B3 clone as a positive control, were cut out of the phage vector, ran out on a 0.7 % agarose gel, and transferred onto nitrocellulose. The *Xenopus* N-cadherin clone was used as a control for a cadherin with a different sequence. At the lowest stringency (37° washes in 1XSSC/0.1% SDS), all 20 lanes were labeled, including the N-cadherin lane (figure 1A). The latter, however, gave a very weak signal in comparison to the other lanes. At the highest stringency (70° washes in 1XSSC/0.1% SDS), there was no detectable signal from the N-cadherin lane (figure 1B). However, the lanes from all 19 positive clones gave very strong signals, suggesting that these clones had sequences which would encode identical or very similar proteins as that of the probe, the 7B3 clone.

C. Generation of nested deletions in the 7B3 clone and sequencing.

A series of nested deletions were generated in both the forward (5' to 3') and reverse (3' to 5') directions. The size of the deletions were determined and the appropriate deletion inserts were picked so

that there was overlap between the sequences read from different sequencing reactions (figure 2). The 7B3 clone was sequenced in both the forward and reverse directions to confirm correct reading of the nucleotides (sequence published in Levine et al., 1994; genebank accession #U04708).

The 7B3 clone was found to have a 2645 b.p. coding region with a high degree of homology with mouse P-cadherin (60%) and E-cadherin (56%), particularly in the cytoplasmic domain (figure 3). More recently, *Xenopus* E-cadherin has also been cloned and sequence comparison reaffirmed that C- and E-cadherins are different members of the cadherin family (Levine et al., 1994).

D. Western blotting using specific antibodies against C-cadherin.

Specific antibodies were raised against the extracellular domain of C-cadherin expressed in E. coli (Dale Apatira). In Western blots from *Xenopus* extracts, the antibodies recognized the 120 kD C-cadherin band but not the 140 kD E-cadherin band, demonstrating that the antibodies are C-cadherin specific (Brieher and Gumbiner, 1994).

IV. Discussion:

To generate a C-cadherin-specific probe and to do functional studies, C-cadherin was cloned from a *Xenopus* oocyte cDNA library. Sequence comparison showed that C-cadherin has high sequence homology to other members of the cadherin family, particularly in the cytoplasmic domain. C-cadherin specific antibodies were also generated and found to detect only the 120 kD protein, corresponding to C-cadherin, but not the 140 kD protein, corresponding to E-cadherin, confirming that C-cadherin was not a degradation product of *Xenopus* E-cadherin. In addition, the antibodies were able to temporarily inhibit reaggregation of dissociated *Xenopus* blastomeres, strongly suggesting that C-cadherin is the primary mediator of adhesion between the blastomeres (data not shown).

Although the 18 other positive clones appeared to have similar or identical sequences to that of C-cadherin, as suggested by Southern blotting, it is possible that they do not all encode the same protein. EP-cadherin is most likely to be identical to C-cadherin but transcribed from a different allele since they diverge in only 4 amino acids (Ginsberg et al., 1991). C-cadherin and U-cadherin/XB-cadherin share 90% sequence homology, but whether they are from different alleles or from different genes is not clear (Muller et al., 1994). Some of the other 18 positive clones may, in fact, have been U-cadherin/XB-cadherin. Although they have similar distribution patterns early in development, they diverge later, suggesting that they are different members of the cadherin family.

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With the C-cadherin clone available and with knowledge about *Xenopus* embryology, it is now possible to do functional studies. Antibody inhibition studies are possible but it is not possible to use this technique to study C-cadherin during development, since the embryos must to be dissociated for antibody accessibility. Two less intrusive means to interrupt C-cadherin function is to destroy the C-cadherin transcript by injecting antisense oligodeoxynucleotides into the oocyte to prevent C-cadherin translation during oocyte maturation or to express dominant negative mutants at high levels in the embryo.

Preliminary evidence suggests that the full length C-cadherin clone can translate functionally active protein. Overexpression of C-cadherin was able to cause lesions on the ectodermal surface of gastrula stage embryos when mRNA was injected into two-cell stage embryos, but not by overexpression of E-cadherin or β-galactosidase (Levine et al., 1994). The mechanism by which overexpression of C-cadherin caused surface lesions is uncertain, but may be due to interruption of normal cadherin mediated adhesion, since both C-cadherin and E-cadherin are expressed by the outer layer of ectodermal cells in the gastrula stage embryo. One possible interpretation is that E-cadherin function is more important for the maintenance of the ectoderm and that the exogenous C-cadherin is competing for catenin binding with E-cadherin to prevent normal E-cadherin function. Another possible interpretation is that cells overexpressing the exogenous proteins are preferentially adhering to each other. In sections from gastrula stage embryos injected with the full length construct and β-

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galactosidase, cells expressing the exogenous proteins, as determined by the blue stain, were often found in clumps. The exact cause of the lesions was not determined but may involve one or both of the possibilities mentioned above.

Figure 2.1. Southern blot analysis for the 19 potential C-cadherin clones using the largest clone (7B3) as a probe. A. Autoradiogram after low stringency washes (37°). B. Autoradiogram after high stringency washes (70°). Lanes 1-19, the 19 potential C-cadherin clones. Lane 20, N-cadherin clone.

A.

1   11   19 N

B.

1   11   19 N

Figure 2.2: The *Xenopus* C-cadherin protein sequence.
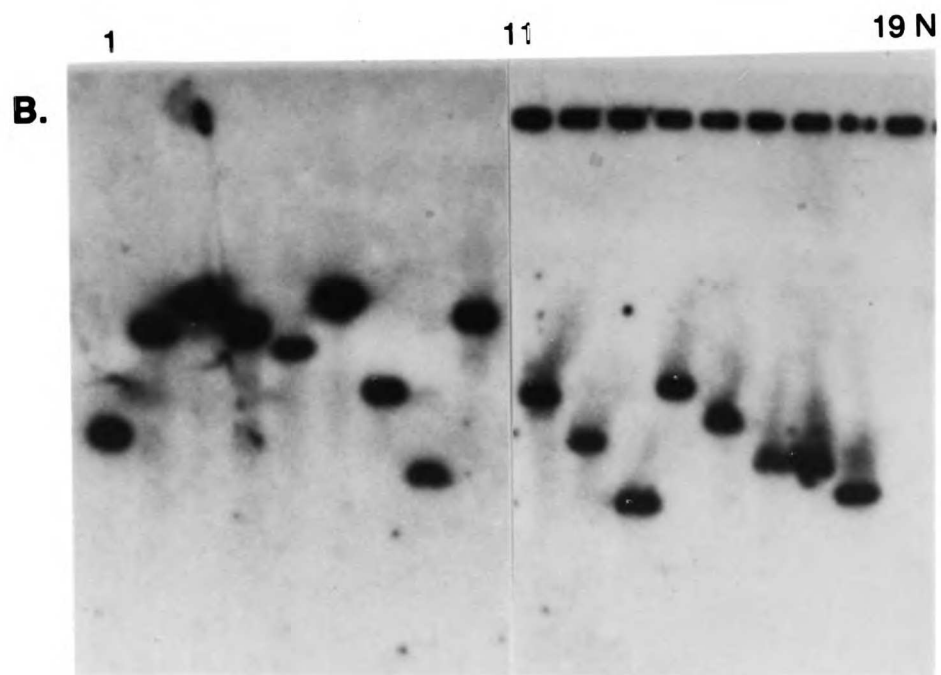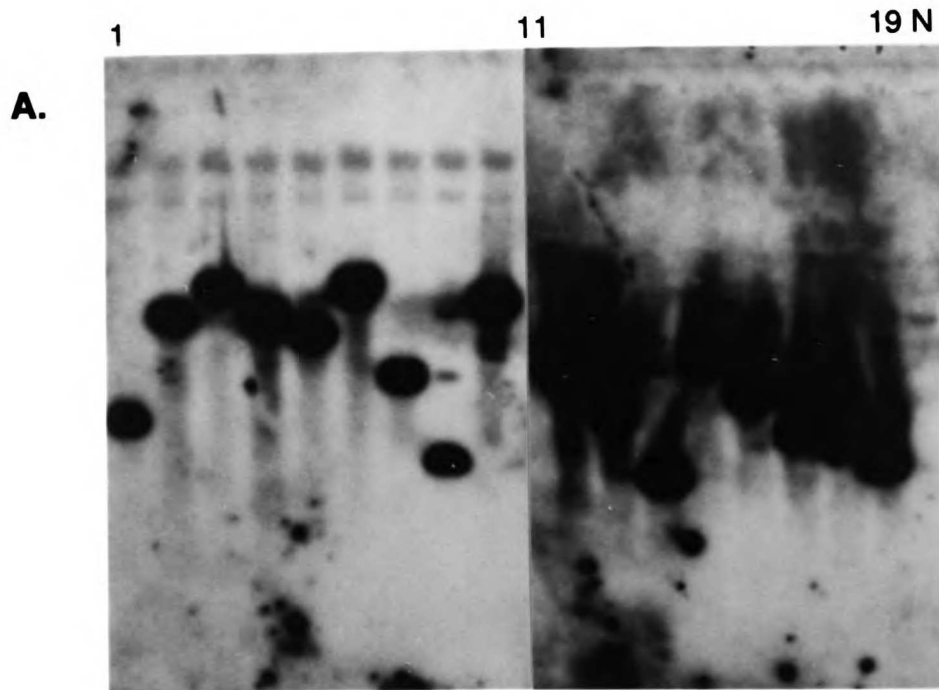
```
            |         |         |         |         |         |         |         |         |         |
           10        20        30        40        50        60        70        80        90        100
  1 MGGTRLRNAS VWLCGLLCLL QVVPSINADV SGCKPGFSSA EYIFSVNRRE LERGRKLGKV NFSDCTTRKH GLYDVGDSRF RVLPDGTVLV KRHVKLHKDT 100
101 KFTISTWDAR GNKHSTNIAV ASKRHRSGEE AHSRSSKLPV LTFPETHTGL KRKKRDWVIP PIKVSENERG PFPKRLVQIK SNKDRFNKVY YSITGQGADN 200
201 PPQGVFRIEW ETGWMLVTRP LDREEYDKYV LSSHAVSENG SPVEEPMEIT INVIDQNDNC PKFTQDVFRG SVREGVQPGT QVMAVSATDE DDNIDSLNGV 300
301 LSYSILKQDP EEPIPNLFTI NRETGVISLI GTGLDREKFP EYTLTVQATD LEGAGLSVEG KAIIQITDAN DNAPIFDPKT YTALVPENEI GFEVQRLSVT 400
401 DLDMPGTPAW QAVYKIRVNE GGFFNITTDP ESNQGILTTA KGLDFELRKQ YVLQITVENA EPFSVPLPTS TATVTVTVED VNEAPFFVPA VSRVDVSEDL 500
501 SRGEKIISLV AQDPDKQQIQ KLSYFIGNDP ARWLTVNKDN GIVTGNGNLD RESEYVKNNT YTVIMLVTDD GVPVGTGTGT LILHVLDVND NGPVPSPRVF 600
601 TMCDQNPEPQ VLTISDADIP PNTYPYKVSL SHGSDLTWKA ELDSKGTSML LSPTQQLKKG DYSIYVLLSD AQNPQLTVV NATVCSCEGK AIKCQEKLVG 700
701 GFDLPIILVI LGSVLALLIL FLLLLFLKR KKVVKEPLLL PEDDTRDNIF DQDYDLSQLH RGLDSRPDIM RNDVVPTLMP APHYRPRPSN 800
801 PDEIGNFIDE NLDAADNDPT APPYDSLLVF DYEGSGSEAA SLSSLNSSNS NDEHDYNYLS DWGPRFRKLA DMYGGDDDEE Z 881
            |         |         |         |         |         |         |         |         |         |
           10        20        30        40        50        60        70        80        90        100
```

31

Figure 2.3. *Xenopus* C-cadherin is homologous to Mouse P-cadherin (60% identity).
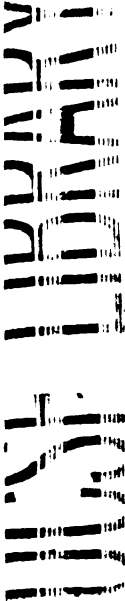
```
C-cad    MGGTRLRNASVWLCGLLCLLQVVPSINADVSGCKPGFSSAEYIFSVNRRELERGRKLGKV
              - signal -

P-cad                                                          MELL


C-cad    NFSDCTTRKHGLYDVGDSRFRVLPDGTVLVKRHVKLHKDTKFTISTWDARGNKFSTNIAV

P-cad    SGPHAFLLLLLQVCWLRSVVSEPYRAGFIGEAGVTLEVEGTDLEPSQVLGKVALAGQGMH


C-cad    ASKRHRUGEEAHSRSSKLPVLTFPETHTGLKRKKRDWVIPPIKVSENERGPFPKRLVQIK
                         :.:..::..::..::: :.::..::::.:: :..:
P-cad    HADNGDIIMLTRGTVQGGKDAMHSPPTRILRRRKREWVMPPIFVPENGKGPFPQRLNQLK
              - pre-region -         |    - N-term ..


C-cad    SNKDRFNKVYYSITGQGADNPPQGVFRIEWETGWMLVTRPLDREEYDKYVLSSHAVSENG
         :::::: .:..::::::.:::.::.::: :: :.::.:. .::::. :: : .:::::::
         SNKDRGTKIFYSITGPGADSPPEGVFTIEKESGWLLLHMPLDREKIVKYELYGHAVSENG


C-cad    SPVEEPMEITINVIDQNDNRPKFTQDVFRGSVREGVQPGTQVMAVSATDEDDNIDSLNGV
         ..:::::.:.:' :.:::::.:::::.:::::.::: ::: ::.:.:::::.... :::
         ASVEEPMNISIIVTDQNDNKPKFTQDTFRGSVIEGVMPGTSVMQVTATDEDDAVNTYNGV


C-cad    LSYSILKQDPEEPIPNLFTINRETGVISLIGTGLDREKFPEYTTVQATDLEGAGLSVEG
         ..::: .:.:.::    .:::::::.::.:.:::::: ::: .:: :.:.: ....
         VAYSIHSQEPKEPHDLMFTIHKSTGTISVISSGLDREKVPEYRLTVQATDMDGEGSTTTA


C-cad    KAIIQITDANDNAPIFDPKTYTALVPENEIGFEVQRLSVTDLDMPGTPAWQAVYKIR-VN
         .:..:: ::::::::: :.:..: ::::.: ::::::.:::::.:. ::.:.:.:  .
         EAVVQILDANDNAPEFEPQKYEAWVPENEVGHEVQRLTVTDLDVPNWPAWRATYHIVGGD


C-cad    EGGFFNITTDPESNQGILTTAKGLDFELRKQYVLQITVENAEPFSVPLPTSTATVTVTVE
         .:. :.:::.::.::.::: ::::::: ..:..: ..:.:..: :::.:::::. :.
         .DGDHFTITTHPETNQGVLTTKKGLDFEAQDQHTLYVEVTNEAPFAVKLPTATATVVVHVK


C-cad    DVNEAPFFVPAVSRVDVSEDLSRGEKIISLVAQDPDKQQIQKLSYFIGNDPARWLTVNKD
         :::::: :::. .... :...: ::   .:::::: ::.:.::..:::..: :
         DVNEAPVFVPPSKVIEAQEGISIGELVCIYTAQDPDKED-QKISYTISRDPANWLAVDPD


C-cad    NGIVTGNGNLDRESE-YVKNNTYTVIMLVTDDGVPVGTGTGTLILHVLDVNDNGPVPSPR
         .: .:..: :::::.: .:::::.:.:.;.::.: :  .::::::.: .  :..:..:.:.::
         SGQITAAGILDREDEQFVKNNVYEVMVLATDSGNPPTTGTGTLLLTLTDINDHGPIPEPR


C-cad    VFTMCDQNPEPQVLTISDADIPPNTYPYKVSLSHGSDLTWKAELDSKGTSMLLSPTQQLK
         ...:.:.: ::::::: :..:: :...:. :... ::.:.:. :.:..:.::.. :: .. ::
         QIIICNQSPVPQVLNITDKDLSPNSSPFQAQLTHDSDIYWMAEVSEKGDTVALSLKKFLK


C-cad    KGDYSIYVLLSDAQNNPQLTVVNATVCSCEGKAIK-CQEKLVGGFDLPIILVILGSVLAL
         ...:...: :::  :. :::..:::::.:. :.  :::   ::: :: :::.::::
         QDTYDLHLSLSDHGNREQLTMIRATVCDCHGQVFNDCPRPWKGGF-----ILPILGAVLAL
                                           |   -. transmembrane -


C-cad    LILFLLLLLFLKRKKVVKEPLLLPEDDTRDNIFYYGEEGGGEEDQDYDLSQLHRGLDSRP
         :.:.: :::.....:. :::::::::::::::::.:::::::::::::::::::..::::::..::
         LTLLLALLLLVRKKRKVKEPLLLPEDDTRDNVFYYGEEGGGEEDQDYDITQLHRGLEARP
                        |          - cytoplasmic tail - .


C-cad    D-IMRNDVVPTLMPAPHYRPRPSNPDEIGNFIDENLDAADNDPTAPPYDSLLVFDYEGSG
         . ..:::::::..:: :::::.::::::::::::: :::.::.::::::::::.:::::::::
         EVVLRNDVVPTFIPTPMYRPRPANPDEIGNFIIENLKAANTDPTAPPYDSLMVFDYEGSG


C-cad    SEAASLSSLNSSNSNDEHDYNYLSDWGPRFRKLADMYGGDDDEEE
         :.:::::::.::.:::.:::::::::.:.:.:.:::::::::...:
         SDAASLSSLTTSASDQDQDYNYLNEWGSRFKKLADMYGGGEDD
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CHAPTER 3
ANTISENSE KNOCK-OUT AND
OOCYTE TRANSFER EXPERIMENTS
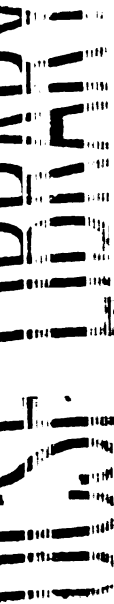
CHAPTER 3:   ANTISENSE KNOCK-OUT/OOCYTE TRANSFER EXPERIMENTS

I. Abstract.

In order to study the function of C-cadherin during early cleavage stages, an antisense approach was worked out to deplete the maternal pool of C-cadherin mRNA. Antisense oligodeoxynucleotides (oligos) were injected into immature oocytes and fertilizable eggs were generated using the oocyte transfer technique. 12 oligos, designed to hybridize to different regions of the C-cadherin transcripts, were screened and two (oligos 96 and 97) were found to induce degradation, as monitored by RNase protection assay. After injection of the oligos, the oocytes were matured in progesterone and the level of C-cadherin was analyzed by western blotting. Very little protein was detected.

A mutant form for C-cadherin was constructed, with deletion of the region corresponding to the oligo (97) hybridization site in the pre/pro domain, to carry out rescue experiments. The mutant was translated at high levels and in a sufficiently correct conformation to be recognized by C-cadherin antibodies raised against the extracellular domain. In addition, overexpression of the mutant was able to cause lesions on the surface of gastrula stage embryos when injected into two-cell stage embryos, much like the overexpression of the wild-type construct (see chapter 2, section IV), suggesting that it was functionally active.

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Out of 400 oocytes used in the oocyte transfer experiment, only 6 eggs, all derived from oocytes injected with the modified form of oligo 97 (m97), fertilized and developed into tadpoles. None of the embryos showed any obvious adhesion defects. The lack of a defective phenotype may have been due to residual expression of C-cadherin, to the presence of other adhesion molecules, or the inhibition of adhesion at the blastula stage with subsequent recovery with zygotic expression (Heasman et al., 1994). The results, however, are inconclusive since the level of C-cadherin was not tested by western blotting, and, more particularly, the sample number was very low.

II. Introduction.

A powerful technique to study the function of a protein during development is to prevent its expression and study the effect. The generation of transgenic and "knock-out" mice has led to a great deal of insight into the function of many mammalian proteins (Robertson, 1987). However, mouse embryos must develop in utero, which makes analysis of proteins expressed early in development difficult, unlike *Xenopus* embryos which can be monitored in a petri dish throughout its development. Although the transgenic approach cannot be used with *Xenopus*, the development of the oocyte transfer technique allows for similar types of analysis to study the function of proteins during early cleavage stages (Heasman et al., 1991). In addition, a great deal of information about *Xenopus* embryology is known in detail, both at the cellular and molecular level (Keller and Winklbauer, 1992; Newport and Kirschner, 1982; Nieuwkoop and Faber, 1967).

One or more cadherins have been speculated to be responsible for maintaining the structural integrity of *Xenopus* embryos, as suggested by the calcium dependent adhesion between *Xenopus* blastomeres (Nomura et al., 1986; Nomura et al., 1988). Cadherins have also been correlated with separation and organization of tissues during morphogenesis (Takeichi, 1991). Although the blastula appears simply as a rapidly cleaving ball of cells, C-cadherin may be involved in the organization of the blastomeres to prepare them for induction and gastrulation movements.

Another intriguing question in the cadherin field is whether each cadherin-type has properties which makes the cell or embryo more adaptable to a particular type of environment. Although both mouse and chicken embryos first express E-cadherin during early cleavage stages, *Xenopus* embryos first express C-cadherin, with E-cadherin expression initiating at the gastrula stage (Choi and Gumbiner, 1989; Choi et al., 1990). Does C-cadherin have any properties which makes it more suitable for the early cleavage stages in *Xenopus*? It is interesting to speculate whether cadherins are interchangable or whether each type has features suited for development in a particular environment.

The generation of C-cadherin "knock-out" embryos will lead to a better understanding of the role of C-cadherin during early cleavage stages. Do the embryos fall apart? Are the embryos cleaving normally? In addition, different cadherins can be used to replace C-cadherin after the knock-out to determine whether the expression of any cadherin is sufficient for normal development.

Unfortunately, we were only able to successfully complete one knock-out/oocyte transfer experiment out of 6. The frog population at UCSF had reached a critically bad stage, where even under the best circumstances, few frogs fertilized and developed normally. None of the oocytes which went through the knock out/oocyte transfer experiment after the first attempt resulted in successful fertilization and development into embryos, due to the additional stress imposed by the experimental manipulation. In addition, we were informed that Janet Heasman and Chris Wylie, who developed the oocyte transfer technique, were also interested in carrying out

the same experiments. They were, in addition, able to successfully generate some C-cadherin knock-out embryos. At this point of study, we decided to take a different approach to learn more about C-cadherin function.
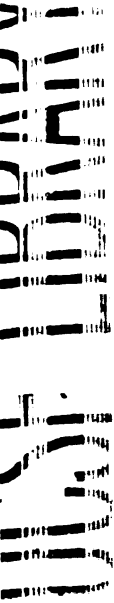
III. Results.

A. Overall strategy for the generation of knock-out *Xenopus* embryos (Heasman et al., 1991).

Oocytes are taken out of adult frogs and separated from follicle cells which form a mesh around each oocyte by manual defolliculation. Fully grown stage 6 oocytes are isolated and the appropriate antisense oligos are injected. The oocytes are incubated in a balanced salt solution, during which the oligos anneal to C-cadherin transcripts and the endogenous RNase H digests the RNA/DNA hybrids. The oocytes are matured in progesterone and transplanted into foster mothers, who lay the experimental eggs along with her own. The jelly coat, which is required for fertilization, is layered onto the oocytes while it is being laid. The eggs are then fertilized and the embryos which develop are examined for mutant phenotype. Figure 1.

B. Expression of C-cadherin in follicle cells.

To screen for antisense oligos which are able to degrade the C-cadherin message, the level of C-cadherin transcripts and proteins were examined by RNase protection assay and western blot analysis, respectively. For the oocyte transfer experiments, the oocytes must be manually defolliculated since exposure to enzymes such as collagenase prevent fertilization (Heasman et al., 1991). However, the oocytes can be isolated using collagenase for screening purposes

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since they do not have to be fertilized for detection of C-cadherin mRNA or protein. Unfortunately, it was discovered that follicle cells also express high levels of C-cadherin and are not completely removed by either the manual or standard collagenase techniques. Although the level of C-cadherin expression in the follicle cells is not important for the transfer experiment, the background C-cadherin signal from the follicle cells must be removed during the screening process to be certain that all C-cadherin transcripts are destroyed by the oligo being tested. Several techniques were examined for removal of follicle cells, as monitored by Hoechst's stain, which illuminates follicle cell nucleus on the surface of oocytes under a fluorescence microscope (data not shown). 1) In the first approach, the oocytes were incubated in calcium-free buffer with collagenase (2 mg/mL). After 5 hours of incubation at room temperature, the follicle cells were completely removed, but the oocytes became more easily damaged. 2) The second approach involves rolling the oocytes on lysine coated slides. Although effective, the drawbacks of this technique were that it was labor intensive, fewer oocytes could be used for each experiment, and the oocytes became very fragile. 3) Finally, the oocytes were incubated in 0.05% trypsin and EDTA (STV), which is used to separate tissue culture cells expressing cadherins. After 30 minutes, no follicle cells were detected on the surface of the oocytes. Furthermore, the oocytes could be matured with progesterone to test for translation of C-cadherin (figure 2). The residual C-cadherin detected after removal of follicle cells may be due to the proteins expressed on the surface of immature oocytes for adhesion with follicle cells .

Nevertheless, when compared to samples where follicle cells were not removed, the level of C-cadherin is much reduced. Whether these proteins are degraded or are recycled for adhesion during cleavage is not known. After exposure to progesterone, the level of C-cadherin increases, suggesting that the STV treatment does not affect translation of C-cadherin during meiotic maturation.

C. Screening oligos/RNase protection assay.

12 oligos were designed and tested for their abilities to anneal to different regions of the C-cadherin transcript and destroy their target (Shuttleworth and Colman, 1988; Dash et al., 1987) (figure 3A,B). The oligos were injected into the oocytes and the oocytes were incubated in 1XMMR for varying lengths of time before total oocyte RNA was extracted and analyzed by RNAse protection assay. The extracted RNAs were mixed with ^{32}P-labeled C-cadherin RNA probe which hybridizes to the C-cadherin transcripts, before RNase A was added to the total mixture (Sambrook et al., 1989). RNase A degrades single stranded RNA, but double stranded RNAs (such as the C-cadherin transcript/probe hybrids) are protected. This whole mixture was run out on an acrylamide gel and exposed to film.

The signal from oocyte extracts depleted of the C-cadherin transcripts is expected to be weaker than in extracts from control uninjected oocytes. Out of the 12 oligos tested, only two were able to deplete the C-cadherin transcripts to a discernible extent. These oligos (96 and 97), when injected at high levels (37-45 ng), lead to depletion of most of the C-cadherin transcripts (figure 4). The level

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of the control EF1α transcripts were not affected by the oligo 109 or water injections, neither of which degraded C-cadherin transcripts to a discernible extent when compared to samples from uninjected embryos, suggesting that the degradation of C-cadherin transcripts by oligos 96 and 97 were specific. However, for the generation of knock-out embryos, the amount of oligos 96 and 97 oligos required for complete degradation is well above what is considered the toxic limit, 25 ng of oligos.

D. Titrating amount of modified oligos and incubation time required to completely deplete C-cadherin transcripts in oocytes.

Oligodeoxynucleotides injected into oocytes are degraded quickly by endogenous enzymes, with half lives as short as 5 min. Modified forms of oligos, however, are available with much longer half lives (Dagle et al., 1990). This permits injection of much lower amounts of oligos, thus reducing toxicity without compromising the level of mRNA destroyed.

Modified forms of oligos 96 and 97 (m96 and m97), as well as control modified oligo against β-galactosidase, were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) in which phosphoramidate linkages were incorporated into the terminal ends of the oligos. Surprisingly, m96 was no longer able to degrade the C-cadherin transcripts after the modification (data not shown).

After injection of 5 ng and 10 ng of m97, the oocytes were allowed to incubate for varying lengths of time to determine the minimal amount of m97 and incubation time required for total

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depletion of the C-cadherin transcripts (figure 5). Total RNA was extracted and RNase protection assay was used to determine the level of the remaining C-cadherin transcripts. With 5 ng of oligo m97, the C-cadherin transcripts were partially depleted by 5 hours and almost completely depleted within 10 hours The level of the control EF1α was not affected by the oligo injection when compared to the uninjected control samples, suggesting that the C-cadherin transcripts were specifically degraded by oligo m97.

E.   Level of C-cadherin in oocytes injected with oligo m97.

To confirm that no C-cadherin transcripts were left to be translated, the oocytes, after injection with oligo m97 and incubation for 9 hours, were defolliculated with STV and matured with progesterone. As was expected, little or no C-cadherin was detected from oocytes injected with oligo m97 by western blot analysis when compared to control uninjected oocytes (figure 6, lanes 1-6 vs. lanes 7-8). Interestingly, injection of higher levels of oligo m97 was not able to deplete the level of C-cadherin any further (figure 6, lanes 1-3 vs. lanes 4-6). In addition, the level of C-cadherin was lower in matured oocytes injected with oligo m97 than the immature uninjected oocytes, suggesting that there might be protein turnover (figure 6, lanes 1-6 vs. lanes 9-10).

F. Rescue mutant construct.

The long term goal of this project was to determine whether different types of cadherins are interchangeable or whether each type has distinctive properties. Should the knock-out prove successful, the aim was to replace C-cadherin with the transcripts for other cadherins to determine whether the expression of any cadherin was sufficient for normal early development.

However, there are several factors to consider. First, the C-cadherin transcripts may be distributed in a regionalized manner, which is not easily reproducible by simple microinjection of transcripts into the oocyte. Second, the level of transcripts in vivo is difficult to estimate, so that replacement with higher or lower amounts of any cadherin transcript, including that for C-cadherin, may lead to abnormal development. Third, the development of the embryos may be altered by the manipulations involved in the generation of the knock-out embryos. Finally, the abnormal phenotype may simply be due to toxicity from the oligo rather than specific degradation of the C-cadherin transcripts.

To demonstrate that changes in phenotype are due to the change in cadherin-type rather than the factors mentioned above, a C-cadherin control is essential to carry out rescue experiments. A mutant form for C-cadherin was constructed with deletion of the oligo 97 hybridization site (mut B), located in the pre/pro domain, which is normally cleaved off during biosynthesis and not required for function. To ensure that this mutant was functional, transcripts for the mutant were injected into embryos at the two cell stage.

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Like the wild-type construct, injection of the mutant construct led to high levels of protein expression and to the development of lesions at the gastrula stage, suggesting that the mutant was functionally active (figure 7) (Levine et al., 1994).

G. Oocyte transfer experiment (in collaboration with Enrique Amaya and Tom Musci).

Pieces of ovaries were pulled out through a small incision made in the ventral body wall from 5 frogs and examined for suitability for the transfer experiment. Over 400 oocytes were manually defolliculated from one batch. 220 oocytes were injected with 5 ng of m97 oligo diluted in 20 nL DEPC-treated water, 70 oocytes were injected with 20 nL DEPC-treated water, and 110 oocytes were left uninjected. The oocytes were cultured in a cooler set at 17-18°C for 6 hours, before progesterone was added to initiate maturation. After another 6 hours, the oocytes were stained with dyes to distinguish the oocytes. A few oocytes showed signs of maturation (white dot in the animal hemisphere, indicative of germinal vesicle breakdown), but the majority did not (data not shown). Two albino frogs, which were injected with HCG 8 hours before, began to lay their eggs. They were anesthetized with tricaine and prepared for surgery. A small incision was made in their body cavity and the experimental oocytes were inserted using a fire-blown glass pipette. After suture, they were transferred into a tank containing 1XMMR and allowed to recover. As the stained eggs were layed, beginning approximately two hours after surgery, they were

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transferred into petri dishes and fertilized immediately with freshly prepared macerated testes. Over a period of 6 hours, 193 stained eggs were collected and 30 embryos were fertilized, as determined by cortical rotation.

14 hours after fertilization of the last batch of eggs, only 8 embryos remained viable. All 8 embryos were derived from oocytes injected with the m97 oligo, and none of the water or uninjected controls survived. By the following morning, only 6 embryos were still viable. Three embryos had kinks in their trunk, but appeared otherwise normal. Two other embryos appeared completely normal, and the last embryo had a defect in closure of the blastopore (data not shown).

IV. Discussion.

The ability to generate RNA "knock-out" embryos in *Xenopus* is a very powerful technique for studying the function of maternally derived proteins (Heasman et al., 1991). Since these proteins are translated during meiotic maturation, which occurs just before the eggs are layed, manipulation to prevent expression of these proteins must occur while the occytes are still immature.

In this study we were interested in investigating the role of C-cadherin in early cleaving *Xenopus* embryos. Is C-cadherin the sole mediator of adhesion? What other functions, if any, does it have? Another interesting question is whether C-cadherin is involved in the physical organization of the blastomeres. Although the blastula appears as a simple mass of quickly dividing cells, the dorsal/ventral axis has been established and the process of mesodermal induction has been in progress for many hours.

To generate C-cadherin deficient *Xenopus* embryos, antisense oligo m97 was used to reduce the level of C-cadherin transcripts in the oocyte. The oocyte transfer technique was used to generate fertilizable eggs from these knock-out oocytes. Since zygotic transcription does not start until the late blastula stage, there was about 9 hours after fertilization to observe the effect due to the loss of function. Out of 400 oocytes injected, only 30 eggs fertilized and 6 developed into tadpoles. No obvious adhesion or developmental defects were observed in the embryos injected with the antisense oligos.

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There are many possible reasons for the lack of a defective phenotype. The most obvious reason is that not all of the transcripts were destroyed by the antisense oligos and enough protein was translated to maintain the integrity of the embryos. Another possibility is that other adhesion molecules are present which may also mediate adhesion between the blastomeres. U-cadherin/XB-cadherin is also known to be expressed early in development, although they comprise only a minor population of the cadherin pool (Muller et al., 1994). Finally, the outer epithelium, which is held together by several adhesion molecules including desmosomes, is able to maintain the integrity of the embryo externally, although the inner cells are affected. This is consistent with the results obtained by Janet Heasman and Chris Wylie, when they generated embryos from oocytes depleted of C-cadherin mRNA (Heasman et al., 1994). They only observe adhesion defects in the internal cells when the outer epithelium is peeled back to exposed the inner cells; in addition, the embryos recovered during gastrulation and developed normally. However, it is not clear if there was complete depletion of the C-cadherin transcripts in their experiments. The results in our experiment are also inconclusive, primarily due to the low sample size.

Very low frequency of fertilized eggs were generated by the oocyte transfer technique. Although it is possible that the oligos made the oocytes and embryos sick, it seems unlikely since the 6 embryos which did develop into tadpoles were all derived from oocytes injected with oligo m97. This bias may be due to an unconscious selection of the healthiest oocytes for the oligo

injections by the injector. The most likely possibility for the low success rate appears to be that the oocytes isolated from different frogs are at different stages of growth and maturity. Although it is difficult to determine visually, this is a crucial factor, since those which are not at the right stage will not fertilized (in this experiment only 30/193 eggs fertilized). The frog's life cycle will have to be followed more closely under a controlled environment to determine the stage at which the oocytes are ready for maturation and fertilization.

Although not easily executed, this approach has the potential to reveal a great deal of information about C-cadherin. The main obstacle appears to be the difficulty in generating large numbers of knock-out embryos. If this line of investigation had been continued, C-cadherin transcripts would have been completely depleted in the oocytes before maturation with progesterone. In addition, western blot analysis and RNase protection assays would have been used to confirm the complete depletion of the C-cadherin transcripts before maturation. Finally, the embryos which did develop would have been fixed at different developmental stages to follow the effect internally.

Figure 3.1. Outline of the oocyte transfer experiment. A. The oocytes are surgically isolated and manually defolliculated. B. Stage 6 oocytes are isolated and injected with oligo m97. C. The oocytes are matured in vitro with progesterone. D. Matured oocytes are transplanted into albino mothers who has started laying eggs. E. Albino frogs will lay both albino and experimental eggs. F. The experimental eggs are collected and fertilized.

A. Surgery and manual defolliculation
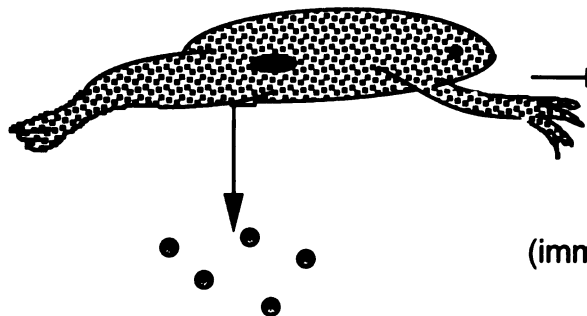
B. Injection with oligo m97
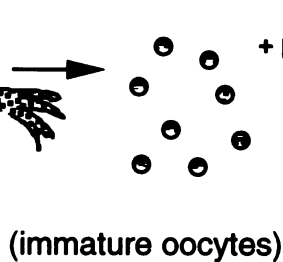
C. In vitro maturation

+ progesterone

(immature oocytes)

(mature oocytes)
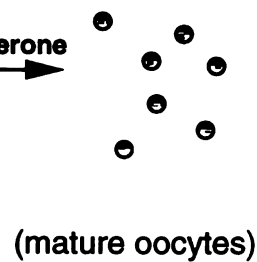
Albino frog induced to start laying eggs with HCG
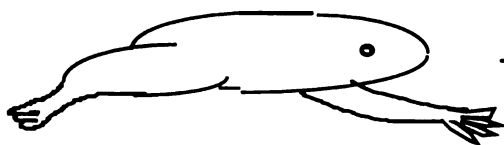
HCG

D. Oocytes transplanted into foster albino female

E. Albino will lay both albino and transplanted eggs

F. Transplanted eggs are collected and fertilized
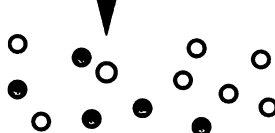
Figure 3.2. Western blot analysis for C-cadherin expression in oocytes and follicle cells. Lanes 1-3, immature oocytes with follicle cells removed. Lanes 4-6, immature oocytes with follicle cells present. Lanes 6-7, matured oocytes with follicle cells removed. Arrow indicates the C-cadherin band.

Figure 3.3. Antisense oligodeoxynucleotides. A. Schematic drawing of the regions on C-cadherin recognized by the oligos. B. List of the oligos and the nucleotides from the C-cadherin sequence recognized by the oligos.

A. Schematic drawing of the regions on C-cadherin recognized by the oligos.
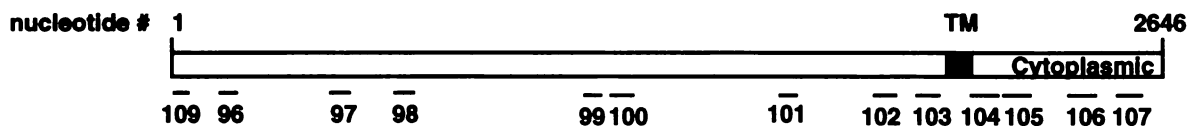
nucleotide # 1   TM   2646

109 96   97   98   99 100   101   102 103 104 105 106 107

B. List of the oligos and the nucleotides from the C-cadherin sequence recognized by the oligos.

Oligos #	Nucleotide #
96	130-146
97	430-446
98	640-656
99	1134-1150
100	1151-1168
101	1646-1661
102	1895-1911
103	1960-1976
104	2124-2143
105	2184-2202
106	2355-2374
107	2427-2448
109	1-17

Figure 3.4. RNase protection assay after injection of antisense oligos 96, 97 and 109 into oocytes. Lanes 1-3, ooocytes injected with 37 ng of oligos 96. Lanes 4-7, oocytes injected with 45 ng of oligo 97. Lanes 8-11, oocytes injected with 45 ng of oligo 109. Lane 12, oocytes injected with H_2O. Lanes 13-14, uninjected oocytes. Thin arrow indicates the control EF1α RNA band. Thick arrow indicates the C-cadherin RNA band.

Figure 3.5. RNase protection assay for modified oligo m97 injections. RNA was extracted at different time points after injection with 5 and 10 ng of oligo m97 to determine the minimal length of time and amount of oligos required to completely deplete C-cadherin transcripts in the oocyte. Lanes 1-2, RNA extracted from uninjected oocytes after 4 hours. Lanes 3-5, RNA extracted from oocytes injected with 5 ng of oligo m97 after 4 hours. Lanes 6-8, RNA extracted from oocytes injected with 5 ng of oligo m97 after 10 hours. Lane 9, RNA extracted from oocytes injected with 5 ng of oligo m97 after 24 hours. Lanes 10-11, RNA extracted from oocytes injected with 10 ng of oligo m97 after 10 hours. Lanes 12-13, RNA extracted from oocytes injected with 10 ng of oligo m97 after 24 hours. Thin arrow indicates the control EF1α RNA band. Thick arrow indicates the C-cadherin RNA band.

	1	2	3	4	5	6	7	8	9	10	11	12	13
Hours (hrs)	4		4			10			24	10		24	
Amount (ng)	0		5			5			5	10		10	
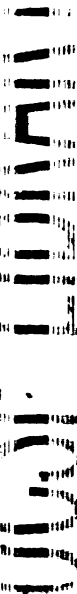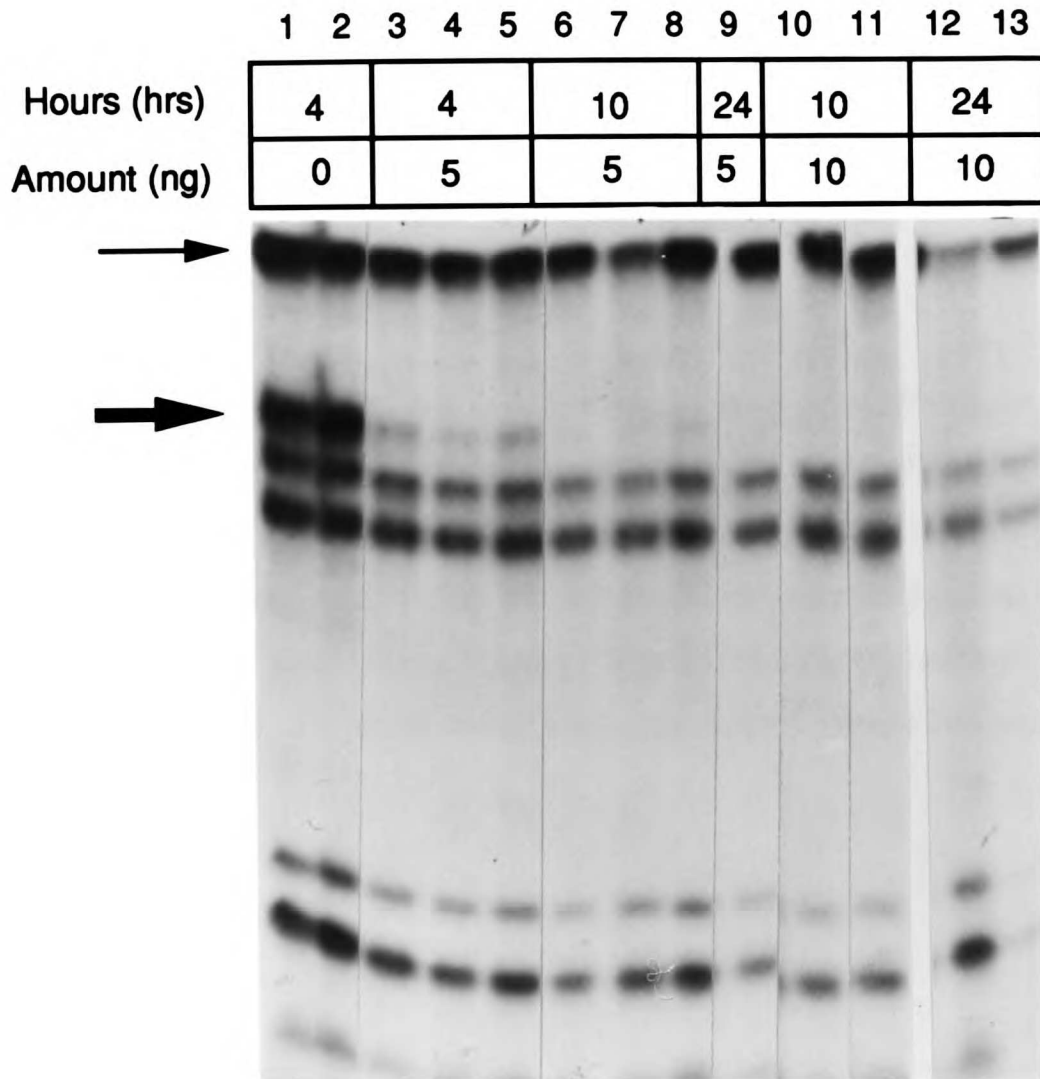
Figure 3.6. Western blot analysis to determine the level of C-cadherin in immature and mature oocytes 9 hours after injection with oligo m97 and defolliculation with STV. Lanes 1-3, oocytes extracted after injection with 12.4 ng of oligo m97 and matured. Lanes 4-6, oocytes extracted after injection with 21.7 ng of oligo m97 and matured. Lanes 7-8, uninjected control matured oocytes. Lanes 9-10, uninjected control immature oocytes. Arrow indicates the C-cadherin band.
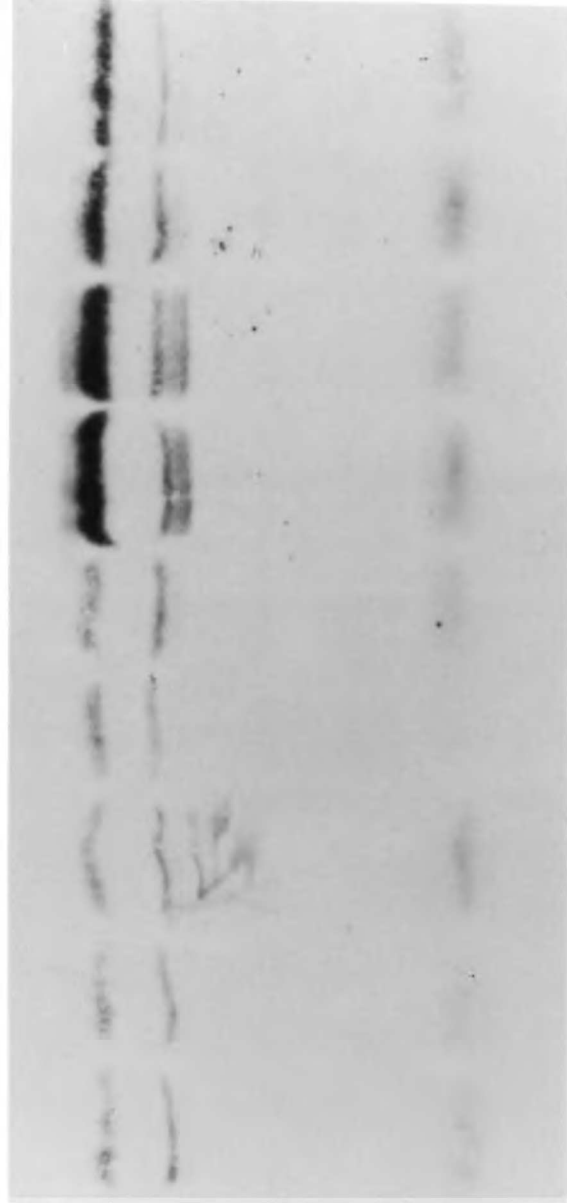
Figure 3.7. Western blot analysis to determine the expression level of wild-type C-cadherin and mut B in *Xenopus* embryos. 3 ng of mRNA was injected into the two-cell stage embryos, total protein was extracted after 4 hours of incubation, and blotted with C-cadherin-specific antibodies. Lanes 1-3, embryos injected with the wild-type construct. Lanes 4-5, embryos injected with mut B. Lanes 6-8, control uninjected embryos. Arrow indicates the C-cadherin band.
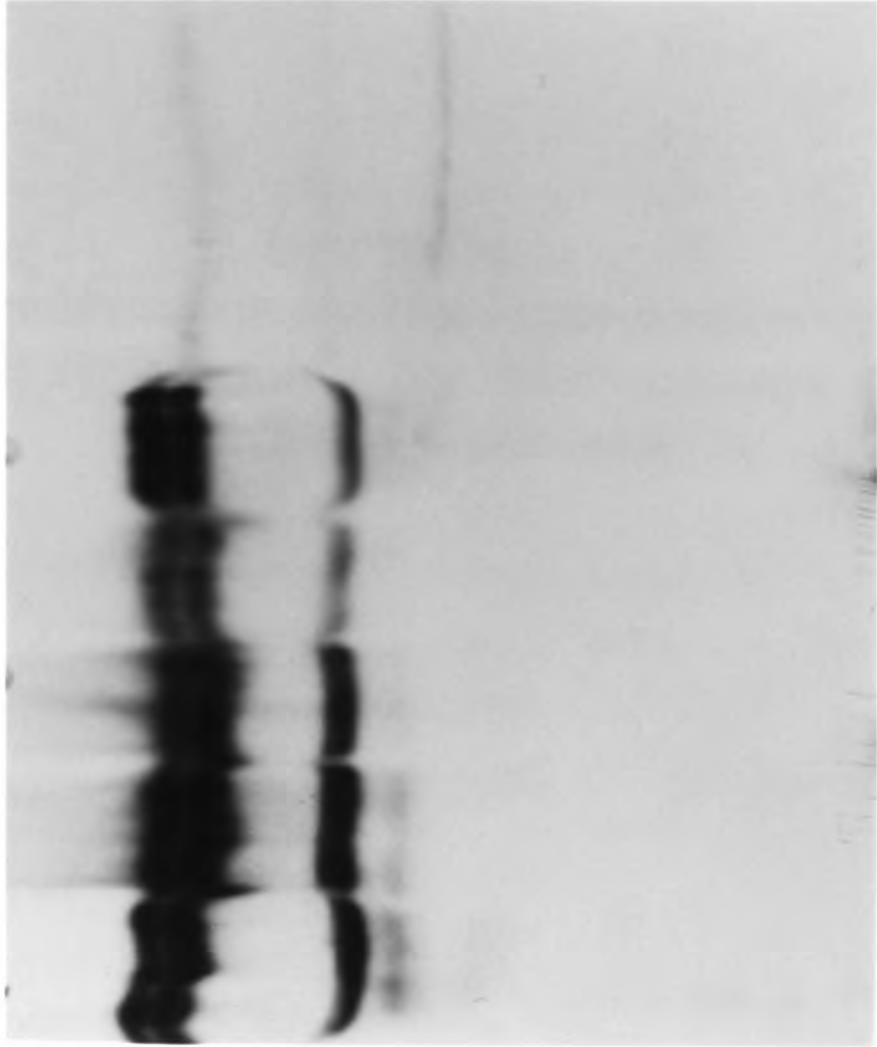
CHAPTER 4

DISRUPTION OF GASTRULATION MOVEMENTS
IN *XENOPUS* BY A DOMINANT NEGATIVE
MUTANT FOR C-CADHERIN

CHAPTER 4: DISRUPTION OF GASTRULATION MOVEMENTS IN *XENOPUS* BY A DOMINANT NEGATIVE MUTANT FOR C-CADHERIN

I. Abstract

Gastrulation in *Xenopus laevis* transforms a ball of undifferentiated cells into a well organized elongated embryo with a distinct body axis as a result of extensive cell rearrangements and movements. Since cell adhesion is thought to play an important role during tissue morphogenesis, we investigated the potential role for C-cadherin, a primary cell-cell adhesion molecule in *Xenopus*, in gastrulation movements. A deletion mutant consisting of the extracellular and transmembrane domains but lacking the cytoplasmic tail (Ctrunc) was constructed to act as a dominant negative inhibitor of the endogenous protein. Injection of in vitro transcribed Ctrunc mRNA into the prospective dorsal involuting marginal zone (DIMZ), the region that undergoes the most extensive movements, caused a reproducible defect in gastrulation. The defect, an inability to complete involution and to close the blastopore, was cadherin specific, because it could be rescued by expressing full length C-cadherin. Ctrunc seems to inhibit morphogenetic movements directly, because it specifically blocked the activin induced elongation of animal cap explants isolated from injected embryos. Expression of Ctrunc did not interfere with inductive processes in the embryos that developed gastrulation defects, because they formed complete axial structures. These results demonstrate that C-cadherin plays a critical role in *Xenopus*

laevis gastrulation movements, and they support the hypothesis that cadherins can mediate cellular rearrangements during tissue morphogenesis.

II. INTRODUCTION

Cadherins constitute the major class of Ca^{2+}-dependent adhesion molecules expressed in solid tissues (Takeichi, 1988, 1991; Geiger and Ayalon, 1992; Grunwald, 1993). Since different populations of cells expressing different cadherins can sort out (Nose et al., 1988), the tissue specific and temporal patterns of cadherin expression are thought to underlie many aspects of histogenesis. Regulation of the expression or function of an individual cadherin type is also important for tissue morphogenesis. For example, in tissues undergoing epithelial-mesenchymal transition and in compaction of mouse embryos, regulation of E-cadherin leads to dramatic changes in cell association (Vestweber et al., 1987; Fleming and Johnson, 1988; Behrens et al., 1989; Ekblom, 1989; Takeichi, 1993).

A particularly interesting morphogenetic process that may involve cadherin function is cell rearrangement (Gumbiner, 1992). Cell rearrangement is a common morphogenetic mechanism occurring in a variety of developing tissues of numerous organisms (Keller, 1987). During cell rearrangement, cells exchange neighbors and reposition themselves, resulting in an overall change in the shape of the tissue. Even though rearranging cells exchange neighbors, they remain closely associated with one another, suggesting an important role for cell adhesion.

We have been interested in the role of C-cadherin (also called EP-cadherin) in the morphogenesis of tissues in *Xenopus laevis* (Levine et al., 1994; Ginsberg et al., 1991). C-cadherin is maternally encoded and is the major cadherin expressed in early cleaving and

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blastula stage embryos. It has been demonstrated to be the primary mediator of intercellular adhesion in the blastula (Heasman et al., 1994) and it continues to be expressed throughout gastrula stage embryos. Moreover, the functional activity of C-cadherin at the cell surface is regulated during experimentally induced morphogenetic elongation of animal caps by activin (Brieher and Gumbiner, 1994), which mimics the convergence and extension movements of the whole embryo. Although the contribution of C-cadherin regulation to tissue elongation is uncertain, it may play a role in controlling morphogenetic movements in the gastrulating embryo.

Convergence and extension by cell rearrangement occurs at the DMZ (dorsal marginal zone), and constitutes one of the major forces underlying gastrulation movements in *Xenopus* embryos (Keller and Winklbauer, 1992). Extensive cell-cell interactions occur between the converging and extending DMZ cells. In this study, we investigate the role of C-cadherin in gastrulation movements at the DMZ, and in the elongation movements of activin-stimulated animal cap explants. We have used a dominant negative approach using a construct encoding the extracellular and transmembrane domain of C-cadherin lacking the cytoplasmic tail (Ctrunc). This construct is similar to the dominant negative mutants that were used to selectively inhibit E-cadherin and N-cadherin functions in *Xenopus* (Levine et al., 1994). The phenotype observed as a result of localized expression of Ctrunc in the DMZ and in animal cap explants implicates C-cadherin in the morphogenetic movements underlying tissue elongation.

III. RESULTS

A. Expression of mutant C-cadherin in the dorsal IMZ induces gastrulation defects.

The mutant C-cadherin (Ctrunc) was designed to act in a dominant negative manner by interfering with the interactions between endogenous C-cadherins. This approach was based on previous work with two similarly constructed mutants for E-cadherin and N-cadherin (Etrunc and Ntrunc), which were found to interrupt cadherin-mediated adhesion (Levine et al., 1994). In vitro transcribed mRNA (4-5 ng) for Ctrunc (Fig. 1) was injected into the prospective involuting marginal zone of a 4-8 cell stage embryo. Western blotting confirmed efficient translation of the injected mRNAs at the expected size (Fig. 6A,B).

Embryos injected with Ctrunc were not able to gastrulate properly (Fig. 2A-D). Unlike embryos injected with control β-galactosidase mRNA, which completed closure of the blastopore by the early neural plate stage (Fig. 2A'-D' and 3B), Ctrunc injected embryos failed to completely close their blastopores (Fig. 2A-D and 3A). From 7 different experiments, an average of 56% of the embryos injected with Ctrunc mRNA (4-5 ng) failed to close their blastopores completely, compared to 0% for embryos injected with β-galactosidase mRNA (4 ng) (Table I). In one experiment not included in table I, 100% of the embryos injected with 5 ng of Ctrunc mRNA showed this gastrulation defect.

The block in closure of the blastopore seemed to occur when cells expressing the mutant protein reached the blastopore lip, where they would normally begin to involute. A small amount of β-galactosidase mRNA was coinjected with Ctrunc to serve as a histochemical marker, and embryos which developed gastrulation defects were fixed and stained with X-gal to localize the area of high protein expression (Fig. 2A-D). The staining was localized to the dorsal side of the blastopore lip (figure 2A,B). Although Ctrunc injected embryos continued to develop, the yolk plug remained exposed even at later stages (figure 2C, arrow). By the tadpole stage, the embryos showed a large opening in the dorsal surface, exposing cells derived from the yolk plug (Fig. 2D). The size of the opening depended on the location of the injection site (not shown). Injection into the more vegetal region of the involuting marginal zone created larger openings since involution was halted earlier; conversely, injection into the marginal zone closer to the animal hemisphere created smaller openings located more posteriorly in the tadpole. Injection of the Ctrunc mRNA anywhere else in the embryo such as the ventral marginal zone, the animal hemisphere, or the vegetal cells, did not cause any defects in gastrulation (data not shown), suggesting that the observed block in involution was due to a specific effect on the cells of the dorsal marginal zone.

Sections through embryos with Ctrunc-induced gastrulation defects confirmed that involution was inhibited when cells expressing Ctrunc reached the blastopore lip (Fig. 3A). Control embryos injected with similar amounts of β-galactosidase mRNA, which were fixed at the same stage, closed their blastopores over

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the yolk plug. There was extensive movement of mesodermal cells expressing β-galactosidase over the lip and across the blastocoel roof (Fig. 3B).

Interestingly, the Ctrunc injected embryos exhibiting gastrulation defects developed normal anterior structures, including a complete head with eyes and cement glands, despite incomplete involution of the marginal zone (Fig. 2D). The induction of anterior structures, therefore, does not appear to depend on the extent of involution.

The morphology of the embryos resulting from injection with Ctrunc mRNA was superficially similar to the ones observed by Amaya et al. when they expressed a dominant negative form of the FGF receptor, which inhibited the induction of ventral mesoderm and the formation of posterior axial structures (Amaya et al., 1991). For example, they observed posterior truncations of the notochord, which they attributed to a block in induction by bFGF. To test whether Ctrunc affected either induction or morphogenic movements directly, we examined notochord development. Embryos with gastrulation defects were fixed and stained in whole mount with a notochord specific antibody, tor 70 (Bolce et al., 1992) (Fig. 4A). Similarly staged control embryos injected with β-galactosidase mRNA alone were also processed in parallel (Fig. 4B). Formation of the notochord was not inhibited in embryos exhibiting the Ctrunc-induced gastrulation defect. Staining was observed from the anterior to posterior end without disturbance, much like the control. Unlike the control embryos, however, the notochords in the mutant embryos were moved to one side of the opening produced by the

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gastrulation defect. Therefore, Ctrunc appeared to cause defects in gastrulation movements without affecting mesoderm induction, unlike embryos expressing the dominant negative form of the FGF receptor, which exhibited defects in gastrulation as a result of inhibiting mesoderm induction.

Ctrunc was predicted to interfere with normal gastrulation movements by reducing cell adhesion. For comparison, we overexpressed wild-type C-cadherin, which should increase adhesion, in the DIMZ. Interestingly, the embryos injected with the wild-type C-cadherin alone also developed gastrulation defects. However, these defects appeared different from Ctrunc injected embryos, both externally and in paraffin sections. Externally, the failure to close the blastopore was more exaggerated, with a larger sized yolk plug protruding from the embryo (Fig. 5A). Sections through effected embryos at early neural plate stage indicated that involution seemed to have been partially inhibited. Nevertheless, the mesodermal tissue overexpressing full length C-cadherin, localized by staining for β-galactosidase, did involute and were able to elongate (Fig. 5B,C). Also, there was less involution on the ventral side compared to embryos injected with β-galactosidase or with Ctrunc mRNA which were fixed at the same stage (Fig. 5B and 3A,B). Additionally, Brachet's cleft, which separates the preinvoluted and postinvoluted marginal tissues, was less delineated in the embryos overexpressing the full length C-cadherin in the DMZ, while the archenteron appeared to be enlarged. Finally, the elongated marginal zone often appeared to press against the yolk plug and to localize more anteriorly towards the animal hemisphere

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(Fig. 5A).   The difference in morphology between the gastrulation defects produced by overexpression of wild-type C-cadherin and those produced by Ctrunc suggests that the defects were produced by different mechanisms.

B.   Rescue of the gastrulation defect with wild-type C-cadherin.

Since the mutant C-cadherin was constructed to act in a dominant negative manner, i.e. by competing with the endogenous C-cadherin, we reasoned that increasing the level of wild-type C-cadherin should rescue any mutant-induced defect.   Embryos that were coinjected with both mutant C-cadherin and wild-type C-cadherin showed a significant reduction in the number of embryos with gastrulation defects, as scored by the ability to complete closure of the blastopore (Table I).   Rescue was never complete because overexpression of full length C-cadherin caused defects on its own (Fig. 5).   In fact, the correct ratio of the two forms of cadherins was critical for the success of the rescue experiment. The amount of wild-type C-cadherin that was able to rescue the Ctrunc-induced gastrulation defects varied from experiment to experiment, perhaps due to the variability in the quality of the injected mRNAs.   In each experiment, therefore, 1, 2, or 4 ng of the wild-type C-cadherin mRNA was coinjected with 4 ng of Ctrunc mRNA to determine the optimal amount for rescue.   For controls, similar amounts of β-galactosidase mRNA were coinjected with Ctrunc.   Western blots for each experiment confirmed the expression of Ctrunc (Fig. 6A).   In particular, Ctrunc was confirmed to be

expressed at the same or higher level when injected with the wild-type C-cadherin as when injected along with β-galactosidase mRNA. From 7 different experiments, a total of 89 out of 158 (56%) embryos injected with Ctrunc and β-galactosidase mRNAs developed defects in gastrulation (table I). When wild-type C-cadherin mRNA was co-injected with the Ctrunc mRNA, an average of 49 out of 199 (25%) embryos showed gastrulation defects, a statistically significant reduction (p<0.003). Therefore, wild-type C-cadherin was able to rescue the defects produced by Ctrunc when used at the appropriate ratio.

C. Animal cap assays

Animal caps treated with the growth factor activin elongate by convergent extension movements much as they occur in the DMZ during gastrulation in the whole embryo. They have often been used as models for mesoderm induction during gastrulation. To investigate whether Ctrunc interferes with tissue movements (convergence and extension) more directly, animal caps were isolated from embryos injected with 1-2 ng mRNA into each of the 4 cells at the 4-8 cell stage and then treated with activin (Fig. 7). A trace amount of β-galactosidase mRNA was coinjected to determine the extent of expression through the tissue. Animal caps from control embryos, injected with 1 or 2 ng β-galactosidase mRNA into each of the 4 cells, elongated normally (Fig. 7A',B'). Elongation of caps isolated from embryos injected with 2 ng Ctrunc mRNA into each of the 4 cells, however, was strongly inhibited (Fig. 7A).

Although the mutant injected animal caps induced by activin still developed lumps and bulges not observed for the uninduced animal caps (compare Fig. 7A to 7C, C'), they did not extend the long protrusions observed in control activin-treated caps. The residual tissue movements observed may have been due to cells not expressing Ctrunc, since many of the cells making up the bulges did not stain with the co-expressed marker β-galactosidase. At lower concentrations of Ctrunc mRNA (1 ng into each of the 4 cells), many of the isolated animal caps were able to elongate somewhat, but the amount of movement was not as extensive as in control β-galactosidase injected embryos (Fig. 7B,B'). When the caps were not exposed to activin, there was no observable difference in phenotype between the Ctrunc and β-galactosidase control injected animal caps (Fig. 7C,C').

Although the expression of Ctrunc mutant inhibited elongation movements, it did not cause the cells of the cap to dissociate, suggesting that the inhibition of movement was caused by a subtle change in cell-cell adhesion. In contrast, embryos injected with a second dominant negative mutant, Ctail, which included only the cytoplasmic tail, caused animal caps to dissociate completely (Fig. 7D). Much like a mutant with a deletion of the extracellular domain of *Xenopus* N-cadherin (Kintner, 1992), whole embryos injected with Ctail also dissociated extensively during early gastrula stage (data not shown). Therefore, inhibition of elongation movements (convergent extension movements) by Ctrunc was probably caused by a more subtle inhibition of C-cadherin mediated adhesion.

D. Adhesion defects produced by expression of Ctrunc in the animal hemisphere of Xenopus embryos.

To confirm that Ctrunc does affect cell adhesion, in vitro transcribed mRNA encoding Ctrunc was injected into the animal pole of one cell at the two cell stage. Injection of 3-5 ng of Ctrunc mRNA into the animal hemisphere was sufficient to cause disruption of adhesion at the gastrula stage (Fig. 8A). Initially, indentations appeared in the ectodermal surface, but the outer cells remained intact (result not shown). Eventually, the indentations developed into disruptions that appeared on the embryo surface at later stages. To examine the primary effects of Ctrunc before disruption of the protective epithelial surface, embryos exhibiting indentations at early to mid-gastrula stage were fixed, stained with the substrate for β-galactosidase (X-gal), and imbedded in paraffin (Fig. 8A). The stained inner cells appeared less adhesive, although the outer pigmented cells remained intact, suggesting that the inner cells were affected before the outer cells. Control embryos injected with β-galactosidase mRNA alone remained intact and showed no perturbation (Fig. 8B). The disrupted epithelium often healed and, although remnants of the disruption remained, the mutant embryos, as well as control embryos, continued to develop normally without interrupting gastrulation movements.

To test the specificity of Ctrunc expression, full length C-cadherin mRNA was coinjected with Ctrunc mRNA to attempt to rescue the adhesion defects. Embryos which were coinjected with both Ctrunc and wild-type C-cadherin showed a significant reduction

in the number of embryos with adhesion defects, as scored by appearance of disruption of the outer pigmented cells at later times (Table II). Similar to the rescue of the Ctrunc-induced gastrulation defects, the appropriate ratio of Ctrunc to wild-type C-cadherin mRNA was critical for rescue. There was a statistically significant reduction of up to 50% in the number of embryos showing defects in adhesion (table II, expt. #1 and #2) and/or a much less pronounced defect (table II, expt. #3) in embryos which were coinjected with Ctrunc and full length C-cadherin ($P < 0.04$).

IV. DISCUSSION

The role of C-cadherin in *Xenopus* gastrulation movements was investigated in this study using a dominant negative mutant consisting of the extracellular and transmembrane domain (Ctrunc). Previous work with similarly constructed mutants for E-cadherin and N-cadherin (Etrunc and Ntrunc) showed that such mutants were active as dominant negative inhibitors of the endogenous cadherins (Levine et al., 1994). Injection of Ctrunc mRNA into the animal hemisphere led to disruption of adhesion in the cells expressing Ctrunc, which could be rescued by coinjecting with the full length C-cadherin, demonstrating the specificity of the phenotype. Paraffin sections through these Ctrunc mRNA injected embryos, which at early stages did not exhibit disruptions of the outer layer of pigmented cells, showed that the inner cells were affected before the outer cells. This was presumably because the integrity of the outer pigmented cells could be sustained longer by the presence of other adhesion elements such as E-cadherin, desmosomes, and tight junctions.

To examine the role of C-cadherin on cells undergoing gastrulation movements, the mRNA for Ctrunc was injected at the 4-8 cell stage into the prospective dorsal involuting marginal zone, which undergoes the most extensive movements. In Ctrunc injected embryos, gastrulation movements were partially inhibited, resulting in the failure of the blastopore to close. Involution appeared to be halted when cells expressing Ctrunc reached the blastopore lip. Since injection elsewhere in the embryo did not lead to gastrulation

defects, movements in the DIMZ appeared to be particularly sensitive to a decrease in adhesion. The gastrulation defects observed with Ctrunc could be partially rescued by coinjecting the wild-type C-cadherin mRNA, demonstrating that the defect in gastrulation movements was specifically due to a decrease in cadherin-mediated adhesion rather than nonspecifically due to cellular toxicity.

The inhibition of gastrulation movements in the embryos and the inhibition of elongation movements of activin-induced animal caps were unlikely due to overt cell dissociation, since they remained intact. In contrast, embryos and animal caps isolated from embryos injected with a second dominant negative mutant encoding just the cytoplasmic tail (Ctail), which is known to more completely block adhesion, dissociated dramatically. This Ctail mutant, like the dominant negative used by Kintner, probably caused a more complete inhibition of C-cadherin mediated adhesion by binding to catenins (Kintner, 1992). In addition, overexpression of full length C-cadherin in the DIMZ also caused gastrulation defects, albeit morphologically distinct from those observed with Ctrunc, demonstrating the importance of proper balance in cadherin-mediated adhesion. The importance of an appropriate level of adhesion in tissue morphogenesis is also supported by the findings that changes in cadherin-mediated adhesion occur in animal caps exposed to the growth factor activin (Brieher and Gumbiner, 1994).

In the whole embryo it is difficult to know exactly which morphogenetic processes are being affected by Ctrunc, because gastrulation involves different cell behaviors by various population

of interacting cells. For example, Winklbauer et al. found that when explants of head mesoderm were dissociated by antibodies against U-cadherin, their normal directional migration on fibronectin was inhibited (Winklbauer et al., 1992). In this case, cell motility on fibronectin was not affected, but dissociation of the cells inhibited the directional migration of the tissue as a whole. Nevertheless, it is likely that Ctrunc inhibits convergence and extension movements of the marginal zone cells rather than migration of the head mesoderm. Involution, convergent extension, and closure of the blastopore can occur in the absence of the blastocoel roof, which is the normal substrate for migration of the head mesoderm (Keller and Jansa, 1992). Similarly, RGD peptides do not block blastopore closure or involution in *Xenopus*, in contrast to their potent effects in *Pleurodeles*, which rely heavily on migration for movement (Keller and Winklbauer, 1992). Finally, elongation of animal cap explants treated with activin, which exhibit primarily convergent extension type movements rather than migration (Symes and Smith, 1987), was also inhibited by Ctrunc.

Ctrunc expression seems to inhibit morphogenetic movements rather than induction. In embryos with Ctrunc-induced gastrulation defects, the formation of the notochord was relatively normal when compared to embryos injected with the β-galactosidase mRNA. This contrasts with embryos injected with the dominant negative form for the FGF receptor, which developed a superficially similar looking gastrulation defect, but also exhibited a striking truncation of the posterior region of the notochord (Amaya et al., 1991). Furthermore, Ctrunc embryos developed normal anterior structures, even though

they did not gastrulate completely. This contrasts with earlier findings using suramin and trypan blue to inhibit gastrulation, in which the amount of anterior structures formed correlated with the extent of tissue movement (Kao and Danilchik, 1991). Although the mechanisms by which these agents act are poorly understood, it is possible that they interfere with induction processes rather than movements per se. Therefore, the gastrulation defects resulting from the expression of Ctrunc appeared to be a result of an alteration in the physical movements, rather than a failure of induction events.

Our findings implicate C-cadherin in convergent-extension movements in *Xenopus* gastrulation. They do not, however, exclude the possible roles of other cadherins that are expressed at the gastrula stage. U-cadherin/XB-cadherin is a more minor maternal caldherin that is present in blastula and gastrula stages (Angres et al., 1991; Herzberg et al., 1991; Muller et al., 1994). The sequence of U-cadherin/XB-cadherin is very similar to C-cadherin (90% identity), and it is possible that they interact in a heterophilic manner. Also, E-cadherin is expressed in the outer epithelial layer of the time of gastrulation (Choi and Gumbiner, 1989; Levi et al., 1991). In fact, we found that a dominant negative E-cadherin with deletion of the cytoplasmic tail, Etrunc (Levine et al., 1994), also produced gastrulation defects when its mRNA was injected into the DIMZ (result not shown). This may be due to the formation of heterophilic interactions between different cadherins (Volk et al., 1987), a possibility which we are investigating. Nevertheless, the results presented in this paper demonstrate the importance of

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cadherin mediated adhesion, and in particular C-cadherin in gastrulation movements.

Our findings suggest that C-cadherin is actively involved in generating tissue movements during gastrulation. It is apparently not sufficient for C-cadherin to simply hold cells together, because inhibition of C-cadherin by Ctrunc to an extent that does not cause cell dissociation can disrupt gastrulation movements. C-cadherin may help provide traction between cells in the tissues that allows them to rearrange with respect to each other (Gumbiner, 1992). Consistent with this hypothesis are observations that the rearranging marginal zone cells extend lamellopodia onto neighboring cells and use them for traction to intercalate between one another (Keller and Winklbauer, 1992; Shih and Keller, 1992). These extensive cell-cell contacts in the converging and extending DMZ may be dependent on C-cadherin. In fact, one possible mechanism by which overexpression of wild-type C-cadherin cause gastrulation defects may be by increasing adhesion between rearranging cells and preventing them from sliding past one another. The role of C-cadherin in generating this type of movement may be analogous to the roles of integrins in cell motility on non-cellular substrate. A well known precedence for the participation of cadherins in motile processes is N-cadherin-dependent motility of neuronal growth cones (Bixby and Zhang, 1990). Therefore, cadherins may generally participate in motile processes during morphogenesis.

Figure 4.1. Mutant C-cadherin constructs used in this study. **CT**, cytoplasmic domain. **EC**, extracellular domain. **TM**, transmembrane domain.

EC

CT

TM

N

N

C

C

Ctrunc
mutant

Wildtype
C-cadherin

Ctail
mutant
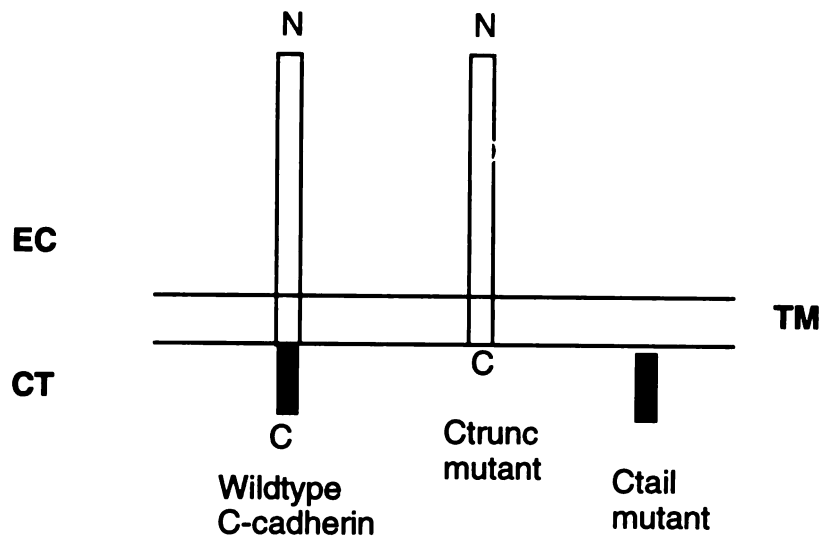
Figure 4.2. Gastrulation defects in embryos expressing the mutant C-cadherin (Ctrunc) mRNA in the dorsal IMZ. A-D) 4-5 ng of Ctrunc mRNA, along with tracer amounts of β-galactosidase mRNA, was injected into the prospective dorsal IMZ at the 4-8 cell stage. A'-D') 4-5 ng of β-galactosidase mRNA was injected at the 4-8 cell stage. A, A') Slit-blastopore stage embryos (stage 13). B, B'). Early neural plate stage embryos (stage 14). C, C') Early tailbud stage embryo (stage 22). Arrow indicates exposed yolk plug. Note that the defective embryos in 2C curls toward the yolk plug. Normal embryos in 2C' are curled laterally because they have not hatched out of their vitelline envelope. D, D') Tailbud stage embryo (stage 37).
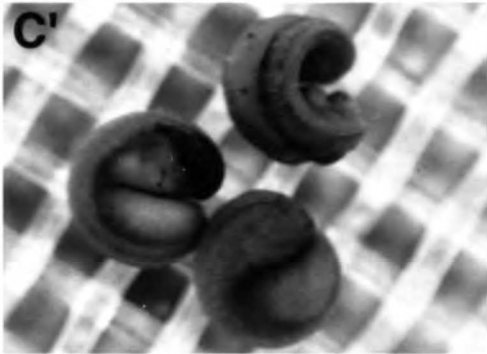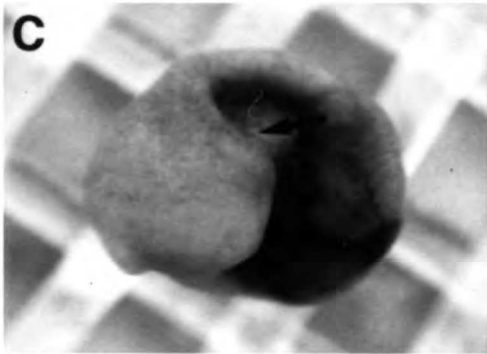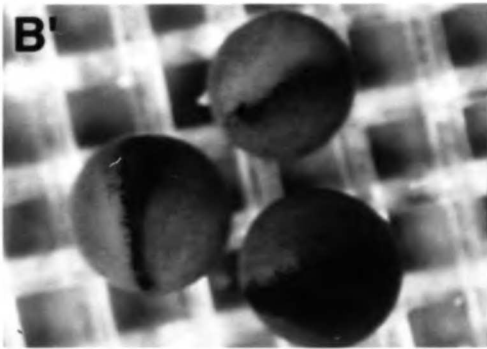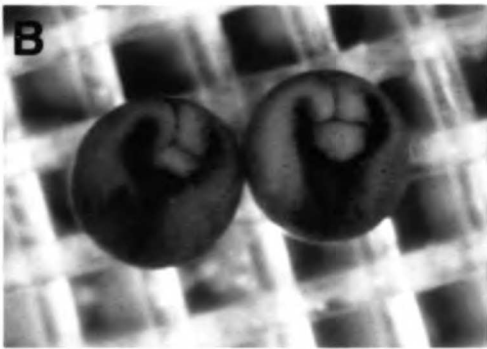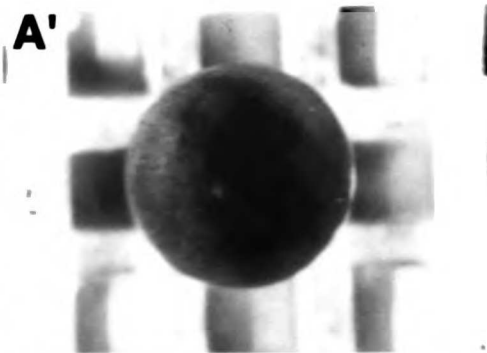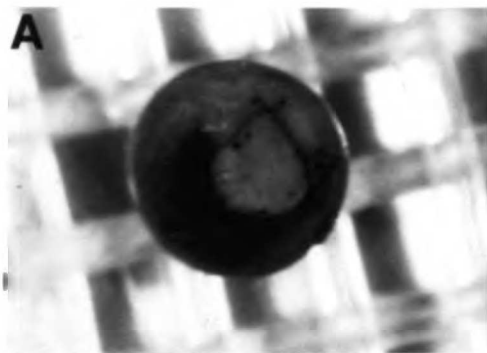
Figure 4.3. Paraffin sections through embryos with gastrulation defects at the early neural plate stage. A) 3.5 ng of Ctrunc mRNA, along with tracer amounts of β-galactosidase mRNA, was injected into the prospective dorsal IMZ at the 4-8 cell stage. B) 3.5 ng of β-galactosidase mRNA was injected at the 4-8 cell stage. **YP**, yolk plug. Arrow points to the dorsal blastopore lip.
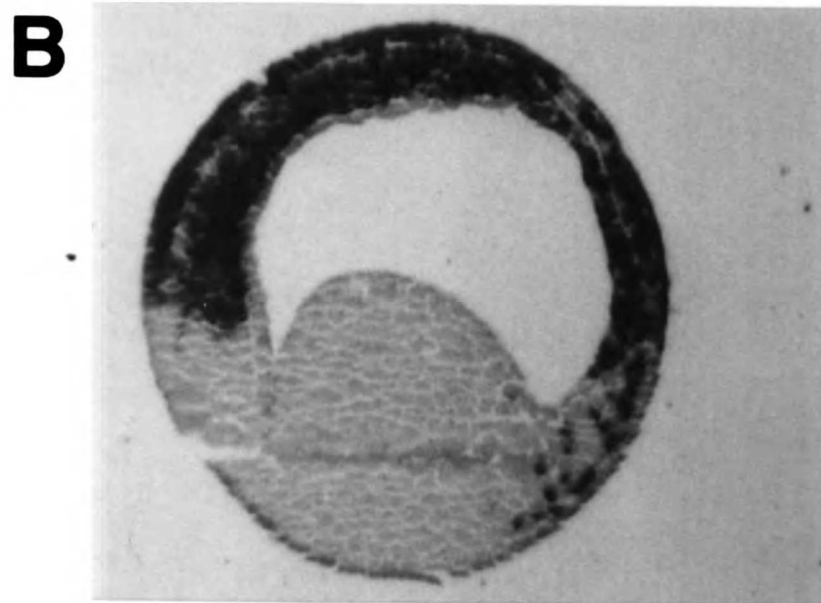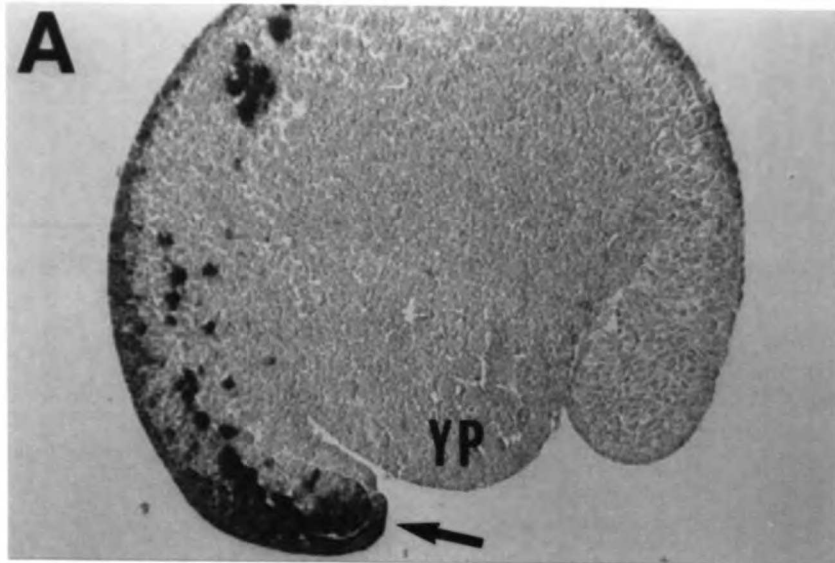
Figure 4.4. Embryos with Ctrunc-induced gastrulation defects develop a complete notochord. Tailbud stage embryos with mutant C-cadherin induced gastrulation defects or control embryos injected with β-galactosidase mRNA were fixed and stained in whole mount with an antibody against the notochord specific antigen, tor 70. A) Ctrunc mRNA injected embryo with a gastrulation defect. B) Control β-galactosidase mRNA injected embryos. Black arrows point to the notochord.

A

B
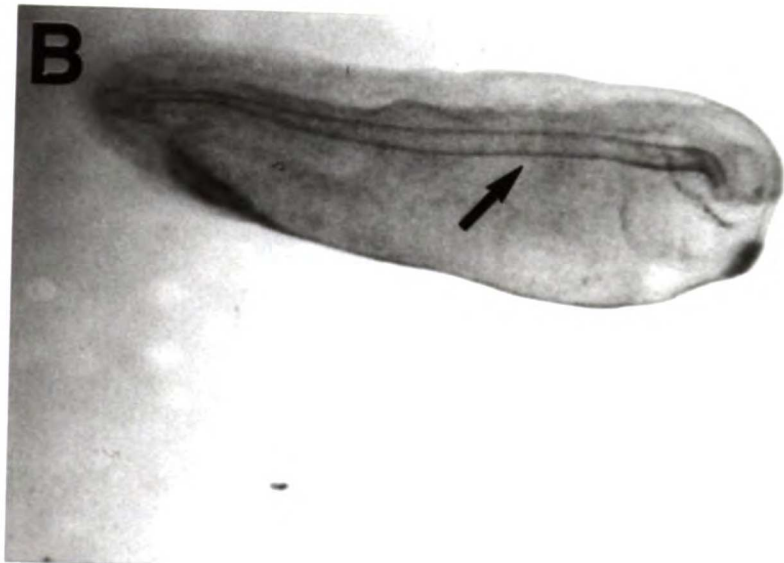
Figure 4.5. Gastrulation defects from overexpression of wild-type C-cadherin. Embryos were injected at the 4-8 cell stage in the prospective DMZ with wild-type C-cadherin mRNA. Low levels of β-galactosidase mRNA were coinjected to determine regions of high expression. A) External view. B) Paraffin section, low magnification. C) Section of another embryo at higher magnification. Arrow indicates location of brachet's cleft. **YP**, yolk plug; **AR**, archenteron.
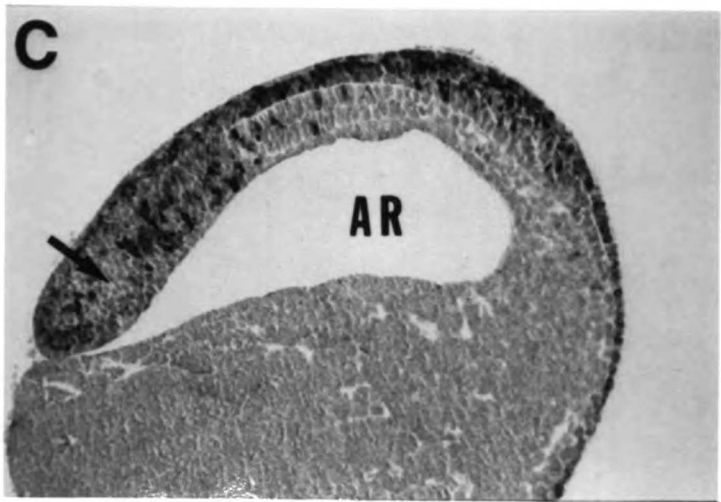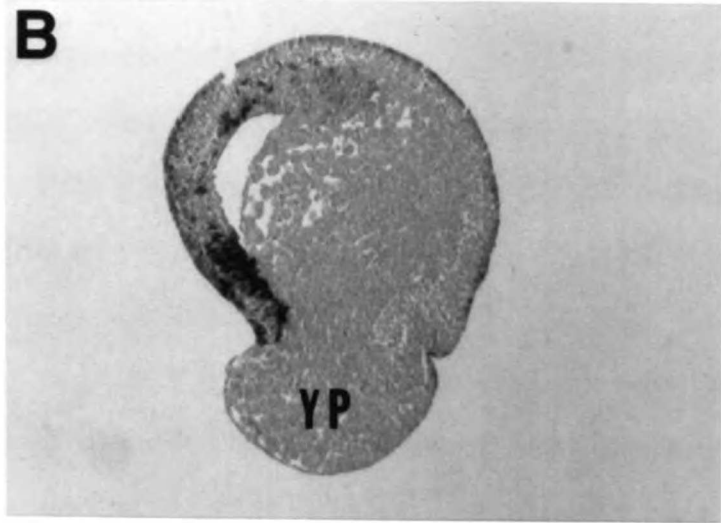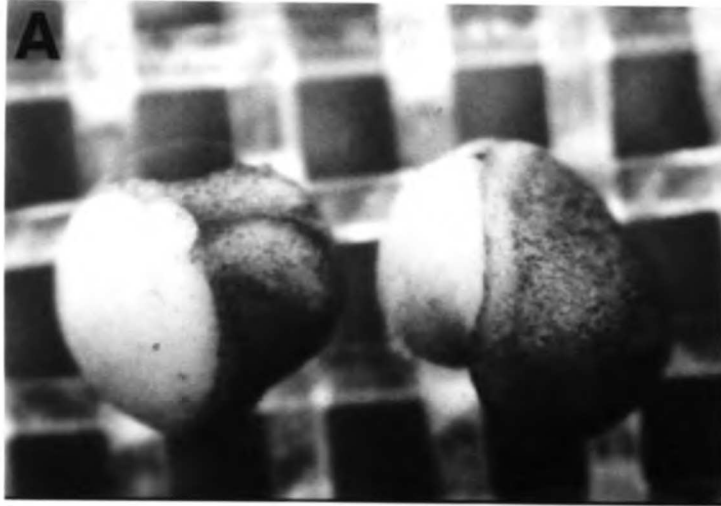
Figure 4.6. Western blot analysis to confirm expression of Ctrunc in both the rescue and control embryos. All samples were loaded in duplicates and immunoblotted with monoclonal antibodies against C-cadherin. A) Rescue of gastrulation defect resulting from Ctrunc injection into the DMZ. Lanes 1-2, 4 ng of Ctrunc mRNA coinjected with 4 ng of the wild-type C-cadherin mRNA. Lanes 3-4, control uninjected embryos for lanes 1-2. Lanes 5-6, 4ng of Ctrunc injected alone. Lanes 7-8, control uninjected embryos for lanes 5-6. B) Rescue of adhesion defect resulting from Ctrunc injected into the animal hemisphere. Lanes 1-2, Ctrunc mRNA coinjected along with wild-type C-cadherin mRNA. Lanes 3-4, Ctrunc mRNA injected alone. Lanes 5-6, uninjected embryos.

A

1,2   3,4   5,6   7,8

C-cadherin →
Ctrunc →
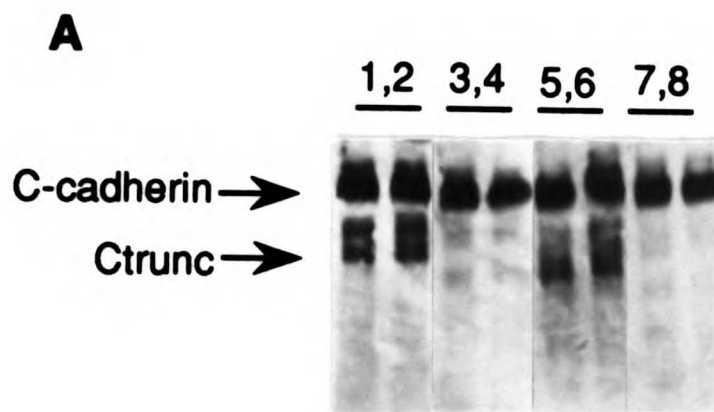
B

1,2   3,4   5,6

C-cadherin →
Ctrunc →
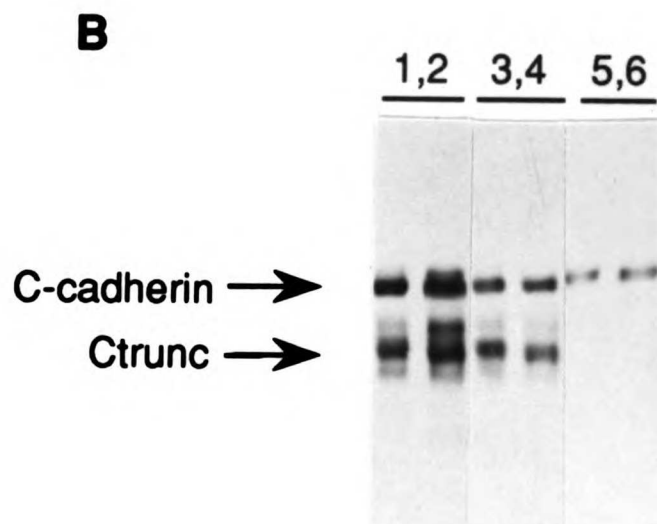
Figure 4.7. Reduction of elongation movements in activin-induced animal caps by Ctrunc. 1-2 ng of mRNA was injected into each of 4 animal hemisphere cells at the 4-8 cell stage. At stage 8, animal caps were isolated and incubated with activin overnight at 20°C. A-C) Animal caps from Ctrunc mRNA injected embryos. A'-C') Animal caps from β-galactosidase mRNA injected embryos. A, A') 2 ng mRNA injected into each of the 4 cells. B, B') 1 ng mRNA injected into each of the 4 cells. C, C') 1 ng mRNA injected into each of the 4 cells, incubated without activin. D) Animal caps isolated from embryos injected with 1 ng x 4 Ctail mRNA, treated with activin.
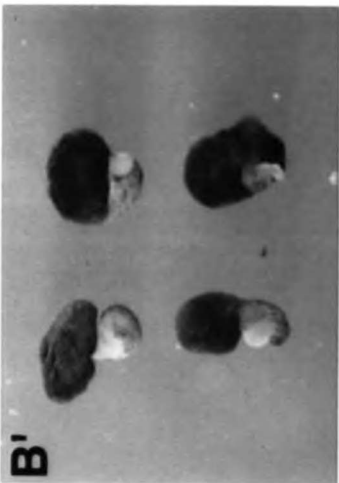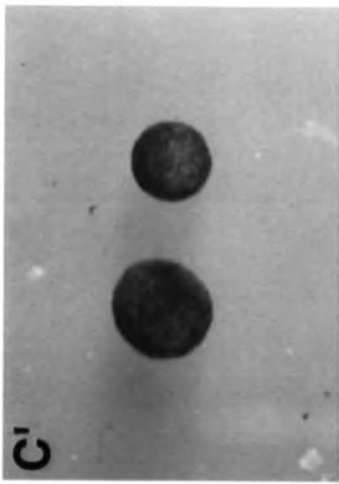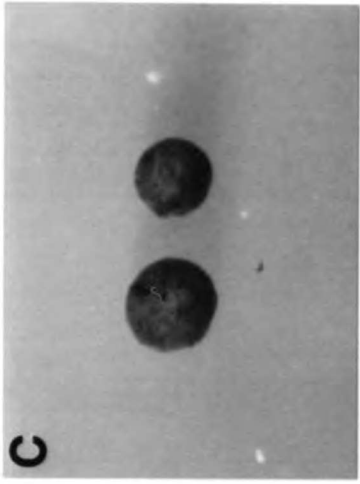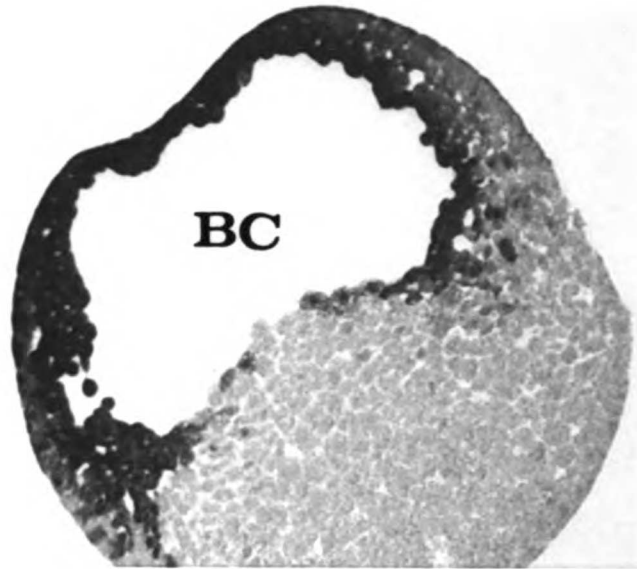
Figure 4.8. Disruption of cell adhesion by injection of the mutant C-cadherin (Ctrunc) RNA into the animal hemisphere. A) Paraffin section of an early gastrula stage embryo injected with Ctrunc mRNA. B) Paraffin section of an embryo injected with β-galactosidase mRNA alone. **BC**, Blastocoel cavity.
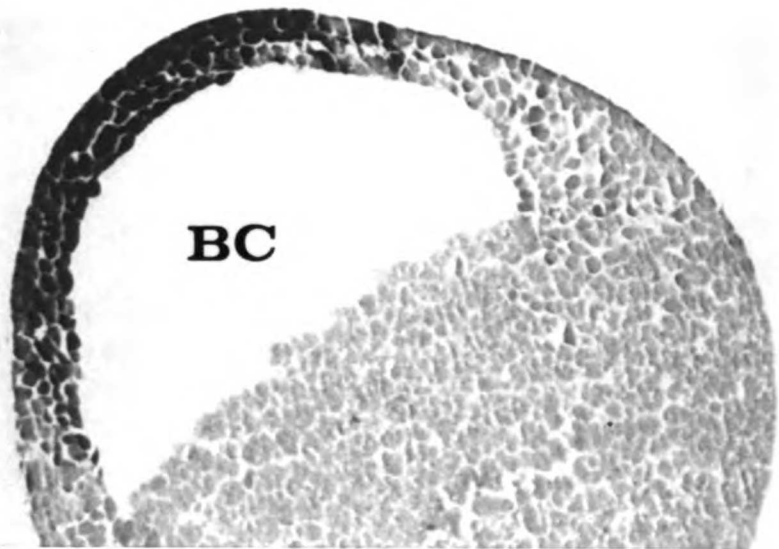
A

BC

B

BC

Table I: Gastrulation defects caused by Ctrunc mRNA injection into the dorsal marginal zone of *Xenopus* embryos, and rescue by wild-type C-cadherin mRNA.

Exp.#	Ctrunc+β -gal mRNA		Ctrunc +wild-type C-cadherin mRNA	
	No. g.d./ total embryos	% defects	No. g.d./ total embryos	% defects
1.	7/19	37	3/26	12
2.	8/32	25	2/30	3
3.	9/22	41	5/35	14
4.	15/33	45	11/33	33
5.	18/29	62	22/40	55
6.	14/23	61	2/20	10
7.	18/28	79	5/15	33
Total	89/158	56	49/199	25
	β-gal alone			
8.	0/14	0		

Gastrulation defects (**g.d.**) were scored as embryos that failed to close the blastopore. β -gal=β -galactosidase mRNA, is a control for RNA injection. 4ng of Ctrunc message was injected in each experiment, with the exception of expt. #7 where 5 ng of mRNA was injected. When full length C-cadherin is co-injected with mutant C-cadherin, there is a significant reduction of gastrulation defects (P<0.003; student's *t* test).

Table II: Frequency of adhesion defects observed by embryos injected with Ctrunc mRNA into the animal hemisphere and rescue by the full length C-cadherin message.

Exp.	Ctrunc + β-gal mRNA		Ctrunc + wild-type C-cadherin mRNA	
	No. a.d*./ total embryos	% defects	No. a.d*./ total embryos	% defects
1.	35/39	90	17/31	55
2.	42/43	98	15/30	50
3.	34/53	64	19/37	51
Total	111/135	82	51/98	52
	β-gal alone			
4.	0/17	0		

Adhesion defects (a.d*) were defined as disruptions appearing on the embryo surface. There is a significant reduction of adhesion defects when mutant C-cadherin is coinjected with full length C-cadherin ($P < 0.04$; student's t test).

CHAPTER 5

MATERIALS AND METHODS

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I. Cloning of *Xenopus* C-cadherin and the generation of the Ctrunc, mut B, Ctail, Etrunc, and Ntrunc deletion mutants.

A cDNA clone encoding *Xenopus* C-cadherin was cloned by screening a *Xenopus* λgt10 oocyte cDNA library (kindly provided by D. Kimmelman) using the region of *Xenopus* N-cadherin cDNA corresponding to the cytoplasmic tail (kindly provided by C. Kintner). The 19 clones obtained were very similar, as determined by restriction mapping and Southern blotting. The longest cDNA (7B3) was subcloned into the bluescript vector at the EcoRI site. A set of nested deletions were generated in both the 5' to 3' and 3' to 5' directions and the 7B3 clone was sequenced using standard methods (Sambrook et al., 1989). The sequence (accession #UO4708; Genbank, National Center for Biotechnology Information) is virtually identical to EP-Cadherin (Ginsberg et al., 1991), with differences in only 4 amino acids. The cDNA was inserted into both the pSP64T and pSP36T vectors for transcription using the SP6 promoter (kindly provided by E. Amaya).

To rescue the effect of depleting C-cadherin transcripts with oligo 97 in the oocytes, a mutant construct (mut B) with the sequence recognized by oligo 97 (nucleotides #430-445) removed in-frame was generated by site-directed mutagenesis (Sambrook et al., 1989).

The dominant negative form for C-cadherin (Ctrunc) was constructed using PCR to insert two stop codons immediately after

the transmembrane domain, which resulted in the deletion of the cytoplasmic tail.

The third C-cadherin mutant (Ctail) contains only the cytoplasmic tail, with deletion of both the extracellular and transmembrane domains. An NcoI restriction site, which includes the start codon ATG, was introduced immediately after the transmembrane domain. Since the start codon for the full length C-cadherin clone also contained an NcoI site, the extracellular and transmembrane domains were cut out using NcoI, the remaining sequence gel purified, and religated.

The *Xenopus* E-cadherin and N-cadherin cDNA were isolated by C. Kintner (Levine et al., 1994; Detrick et al., 1990). The dominant negative forms for both E-cadherin and N-cadherin (Etrunc, Ntrunc) were constructed as described previously (Levine et al., 1994).

II. Southern blot analysis.

The 19 potential C-cadherin clones were cut out of the phage vector with EcoRI and run out on an agarose gel. The DNAs were transferred onto nitrocellulose and incubated in formamide prehybridization solution (5X SSC, 5X Denhardt, 50% formamide (w/v), 1% SDS, and 100 µg/mL denatured salmon sperm DNA) for 5 hours at 68°C using standard methods (Sambrook et al., 1989).

The 7B3 clone was cut out of bluescript using EcoRI, run out on an acrylamide gel, and gel purified using geneclean (BIO 101, Vista, CA, 92083). A ^{32}p-dATP-labelled 7B3 probe was generated by random oligonucleotide priming and purified using a spin-column.

The probe was added to the prehybridization mixture and rocked overnight at 68°C. The nitrocellose was washed (1X SSC, 0.2% SDS) first under low stringency at 37°C and exposed to film for 2 hours (Sambrook et al., 1989). The same nitrocellulose was washed again (1X SSC, 0.2% SDS) under high stringency at 68°C and reexposed to film for 24 hours.

III. RNase protection assay.

RNAs from 1-5 oocytes, eggs, or embryos were extracted with 200 μl of ice cold RNA homogenization buffer (300mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, and 1% SDS). The samples were immediately extracted with an equal volume of phenol/chloroform, two times. The RNA was then precipitated with 1 mL ethanol and stored at -80°C until use.

The ^{32}P (α-^{32}P-UTP) labelled RNA probe was transcribed with the T3 RNA polymerase using standard methods (Sambrook et al., 1989) from a 620 nucleotide C-cadherin clone generated by one of the nested deletions for sequencing (bluescript vector). Approximately half the probe recognizes the pre/pro domain and the other half recognizes the first cadherin repeat of the C-cadherin clone.

The RNAs extracted from the oocytes, eggs or embryos were resuspended in 30 mL hybridization buffer (4 parts formamide and 1 part stock buffer (200 mM PIPES, pH 6.4, 2 M NaCl, and 5 mM EDTA)) containing the probe at approximately 5×10^5 cpm. The mixture was heated to 85°C for 5 min., before being transferred to a 45°C bath for 6-16 hours.

350 μL of ribonuclease buffer (10 mM Tris-Cl, pH 7.5, 300 mM NaCl, 5 mM EDTA, and 40 μg/mL ribonuclease A) was added to the hybridization mixture above and incubated for 60 min. at 30°C. 10 μL of SDS (20%) and 2 μL protease K (25 mg/mL) was then added and incubated for another 15 min. at 37°C. The whole mixture was extracted 1X with phenol/chloroform and precipitated with 1 mL ethanol. The samples were then resuspended in 2 μL of TE, mixed with 2 μL of RNA running buffer, heated for 3 min. at 85°C, run out on a small sequencing gel, and exposed to film (Sambrook et al., 1989).

IV. RNA synthesis.

Capped mRNA for injection was generated in vitro using the SP6 RNA polymerase. The concentration was determined by reading the absorbance at 260 nm and the size and homogeneity of the transcripts were examined on a denaturing formaldehyde agarose gel (Sambrook et al., 1989).

RNA was diluted with DEPC-treated water and injected in 10-20 nl volumes. A small amount of β-galactosidase mRNA was also included in most of the injections for histochemical detection or in larger amounts for control RNA injections.

V. Handling of Xenopus eggs and embryos

Egg laying was induced with the hormone human chorionic gonadotropin (Sigma) and the layed eggs were artificially fertilized with macerated testes (Newport and Kirschner, 1982). The eggs

were dejellied with 2% cysteine, pH 8 and rinsed/incubated in 0.1X
MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.5 mM
Hepes, pH 7.8, and 0.01 mM EDTA). For injections, the dorsal side of
the embryo was distinguished from the ventral side by the lighter
pigmentation and the smaller sized cells. During the injection and
for 30 minutes to 2 hours after injection, the embryos were kept in
1X MMR containing 6% ficoll and penicillin/streptomycin to aid in
healing. The embryos were then rinsed and returned to 0.1X MMR.
Embryos were kept at 14°-20°C and staged according to the normal
table of *Xenopus laevis* development (Nieuwkoop and Faber, 1967).

Animal caps were dissected from stage 8-9 embryos and
incubated at 20°C overnight in 1X MMR, with or without human
recombinant activin A at a final concentration of 5 ng/mL (a gift
from Genentech, South San Francisco, CA). The animal caps were
fixed and stained as described below (Kelly et al., 1991).

VI.   Immunocytochemistry and histology.

Embryos were prepared for whole mount immunocytochemistry as
described previously (Hemmati-Brivanlou, 1989). Notochord was
stained with the monoclonal antibody tor 70 (Bolce et al., 1992).

For histology, embryos were fixed, stained with X-gal, imbedded
in paraffin, and sectioned/counterstained with hemotoxylin-eosin
(Kelly et al., 1991). The sections were cut at a 8-10 μm thickness.

VII. Western Analysis

1-5 embryos or oocytes were extracted with 10-40 µl of 1% NP40 in solution A (20mM Hepes, pH 7.4, 150 mM NaCl, 2 mM CaCl) and spun at 13,000 rpm for 15 minutes at 4°C in a microfuge. The supernatant was isolated using a gel-loading pipette tip to avoid the insoluble pellet and the floating lipid fraction. The samples were then mixed 1:1 with sample buffer, boiled, loaded directly on 7% SDS-polyacrylamide gels, and transferred onto nitrocellulose. Immunoblotting was performed by standard procedures, using milk (5% nonfat milk powder, 0.2% Triton X-100 in PBS) to block non-specific binding. To detect *Xenopus* C-cadherin, hybridoma supernatant for monoclonal antibodies 5D5 or 6B6 was used directly or at a 1:1 dilution (Brieher and Gumbiner, 1994). To detect E-cadherin, monoclonal antibody 5D3 was used at a 1:50 dilution (Choi and Gumbiner, 1989). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG detected by enhanced chemiluminescence (ECL; Amersham).

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