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Structure and Distribution of N-Cadherin in Developing Zebrafish Embryos: Morphogenetic Effects of Ectopic Over-Expression

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ABSTRACT **N-cadherin cDNA was cloned from a zebrafish embryonic cDNA library. Analysis of the deduced amino acid sequence of this molecule (ZN-cadherin) revealed a high degree of homology to N-cadherins of other species, except that its pre-sequence is considerably shorter. Nevertheless, following transfection into chinese hamster ovary (CHO) cells, the expressed protein was functionally active, namely participated in calcium-dependent intercellular interactions. Moreover, ectopic over-expression of ZN-cadherin, following mRNA microinjection into 2–4 cell embryos, caused microaggregation and uneven segregation of deep cells, resulting in distorted embryos. Developmental Northern and Western blot analyses indicated that both the mRNA and the protein first appear at gastrulation. In-situ hybridization showed that ZN-cadherin mRNA was initially present in all deep cells, and later became restricted to various epithelial and neural tissues. Whole-mount immunostaining indicated that while ZN-cadherin was already present at 50% epiboly, it became associated with cell junctions only 4–5 h later. In developing somites ZN-cadherin expression was prominent but transient. High levels of the protein were detected in epithelial somites and its expression was apparently down regulated concomitantly with the onset of myogenesis.** © 1994 Wiley-Liss, Inc.

Key words: N-cadherin, Cell adhesion, Zebrafish development, Adherens junctions, RNA microinjection

INTRODUCTION

The cadherin superfamily contains multiple homologous members which display tissue diversity and distinct binding specificities (Cunningham and Edelman, 1990; Takeichi, 1990; Geiger and Ayalon, 1992). These molecules were shown to mediate cell-cell contact in adherens type junctions, thus playing a key role in cell segregation and positioning, and in the control of cell movements and morphogenesis (Geiger, 1989; Albelda and Buck, 1990; Geiger and Ginsberg, 1991; Edelman, 1993). Cadherins consist of a large extracellular domain which contains several inter-homologous repeats, and a highly conserved cytoplasmic domain. Both domains of the cadherin molecule are essential for effective cell adhesion, as shown by transfection of deleted cadherin molecules into cells (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990b; Fujimori and Takeichi, 1993). Cadherins are believed to interact in a homophilic fashion, and their intracellular moieties bind to the plaque proteins catenins, and, through them, to the microfilament network (Ozawa et al., 1989; Kemler, 1993).

Many of the studies on the functions and cellular interactions of cadherins were based on transfection of cultured mesenchymal cells with the respective cDNA. Such transfections were shown to cause differential segregation of cells expressing distinct cadherins, and usually induce epithelialization (Nose et al., 1988; Matsuzaki et al., 1990). Conversely, addition of anti-cadherin antibodies to cultured cells leads to dissociation of intercellular contacts and disruption of their microfilament system (Hatta et al., 1985; Volk and Geiger, 1986; Ozawa et al., 1990a). Moreover, microinjection of cadherin-specific anti sense oligonucleotides into embryos, inhibited the formation of intercellular contacts (Heasman et al., 1994).

One of the most extensively studied cadherins is N-cadherin, which was first identified in the nervous system (Hatta et al., 1985; Hatta and Takeichi, 1986). Its expression during development is closely correlated to morphogenetic processes, such as mesenchyme-to-epithelium transition and folding of epithelial sheets (Hatta et al., 1987; Duband et al., 1988). The functional properties of this molecule were first characterized in transfected cultured cells (Hatta et al., 1988) and recently, its morphogenetic capacity was examined in vivo in *Xenopus* embryos following N-cadherin mRNA injection. This manipulation perturbed normal development, leading to the formation of excessive epithelial structures (Detrick et al., 1990; Fujimori et al., 1990).

As a model system for studying the involvement of N-cadherin in embryonic morphogenesis in vivo, we have chosen the developing zebrafish (*Danio rerio*). These embryos were chosen mainly since they are essentially transparent, develop rapidly and extracor-

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porally, and are thus amenable for continuous microscopic monitoring and manipulation (Kimmel, 1989). In addition, different molecular genetic approaches are currently developed for the isolation of mutants and for experimental manipulation of gene expression in the fish (Westerfield et al., 1992; Mullins and Nusslein-Volhard, 1993).

To set the ground for the study of N-cadherin function in vivo we have cloned and sequenced the zebrafish homolog of this adhesion molecule (ZN-cadherin). We demonstrate that this molecule is localized in cell-cell junctions in transfected mesenchymal cells, and examine its spatio-temporal expression and organization during embryonic development. Moreover, we show that ectopic and premature expression of the protein, following mRNA microinjection to early embryos, affects the distribution of the deep cells, leading to severe perturbation of normal development.

RESULTS

Primary Structure of Zebrafish N-Cadherin

Of 5×10^5 colonies originally screened from the zebrafish cDNA library, three putative N-cadherin clones were selected and subcloned into Bluescript plasmid (Fig. 1). Sequence analysis indicated that the three clones are identical throughout the overlapping regions and revealed an open reading frame of 2343 bp, encoding a 781 amino acid protein. The predicted amino acid sequence is of a characteristic cadherin (Figs. 1 and 2) with four extracellular repeats (EC1-4), a cysteine-rich domain (EC5), a transmembrane region, and a cytoplasmic tail. There is a high degree of homology (80–90%, depending on the region along the molecule) between the zebrafish protein (ZN-cadherin) and N-cadherins of other species including *Xenopus*, chicken, mouse, bovine, and human. In contrast, ZN-cadherin has only 30–60% homology to other cadherins including E-cadherin of different species, P-cadherin, and EP-cadherin (data not shown). Among the conserved sequences are the three paired putative calcium binding sites (Ringwald et al., 1987), the LDRE repeats, the HAV sequence in the N-terminal ectodomain, the four cysteine residues in EC5, the transmembrane region, and the cytoplasmic domain (Fig. 2; for review see Geiger and Ayalon, 1992). One apparent difference between the particular ZN-cadherin cDNA described here, and those encoding N-cadherins of other species, is its relatively short pre-sequence consisting of only 105 bp, between the first AUG and the codon encoding the N-terminus of the mature protein (compared to 300–450 bp in other species). This region contains the conserved endoproteolytic cleavage site, required for proper processing of the protein (Ozawa and Kemler, 1990).

A detailed comparison of N-cadherins of different species was performed by aligning the six known N-cadherin sequences mentioned above, and calculating the degree of interspecies identity at each position. This value was marked as percent identity in Figure 3.

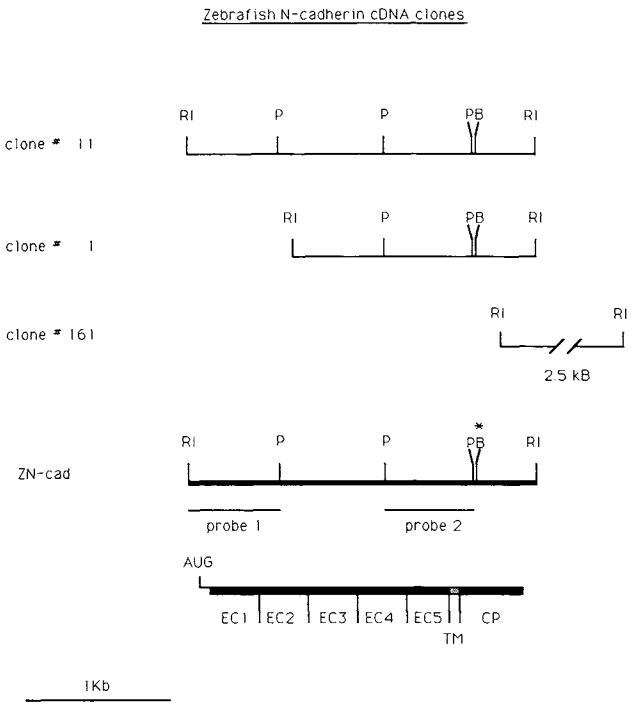

Fig. 1. Restriction maps of the cDNA clones encoding zebrafish N-cadherin (clones #11, #1, and #161). ZN-cad marks the full-length cDNA constructed by ligation of segments derived from clones #11 and #1 (the asterisk indicates the *BamHI* site used to create this clone). Underlined are the two cDNA fragments used as probes for Northern blot analysis and in situ hybridization. A schematic model of ZN-cadherin protein, showing the various cadherin domains including ectodomains 1–5 (EC1-5), the transmembrane (TM), and the cytoplasmic (CP) domains, is shown at the bottom. The restriction sites marked include: *EcoRI*:(RI); *PstI*:(P); *BamHI*:(B).

The positions in which N-cadherin of only one species deviated from the "consensus" were marked in the upper panel of Figure 3. As shown, single amino acid changes were most frequently found in the zebrafish protein (62.4% of all single changes), compared to the other vertebrates (23.3% in *Xenopus*, 7.5% in chick, and 1–2% in the mammals).

Organization of ZN-Cadherin in Transfected CHO Cells

In order to test the capacity of ZN-cadherin to mediate cell adhesion, we co-transfected CHO cells with the respective cDNA, together with the pSV2-neo vector. Western blot analysis of neomycin-resistant clones revealed a $116 \times 10^3 M_r$ band, that was absent from normal CHO cells (Fig. 4). Immunofluorescence staining indicated that the expressed ZN-cadherin was associated with regions of cell contact (Fig. 4B), suggesting that the molecule participates in Ca^{2+}-dependent adhesive interactions. This was further verified by a short (2–10 min) treatment of the transfected cells with EGTA, which resulted in the dissociation of cell-cell junctions (Fig. 4C,D).

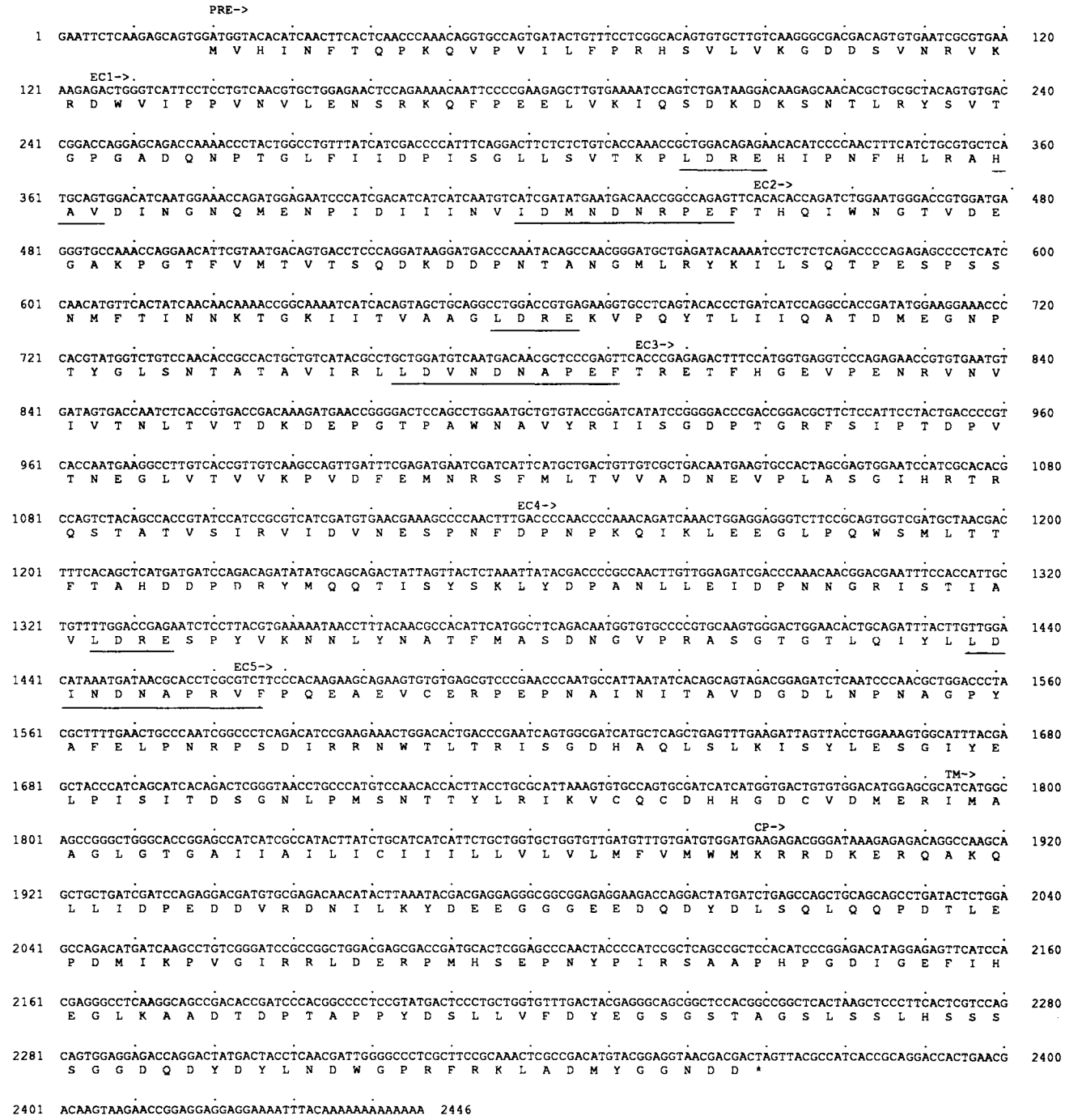

Fig. 2. Nucleotide and deduced amino acid sequences of zebrafish N-cadherin. The protein domains marked above the sequence include the pre-sequence (PRE), ectodomains 1–5 (EC1-5), the transmembrane (TM), and the cytoplasmic (CP) domains. Typical conserved cadherin sequences are underlined including the LDRE repeats, the putative Ca^{2+} binding sites, and the HAV domain (see text). The sequence has been deposited in the GenBank database under the accession number X67648.

Expression of ZN-Cadherin During Zebrafish Development

The expression of ZN-cadherin protein in different embryonic stages was studied using the specific antibody R-851. This antibody recognized a single immunoreactive band of 116×10^3 M_r in extracts of embryos from 50% epiboly and older (Fig. 5). In contrast, the pan-cadherin antibody R-156 detected two distinct

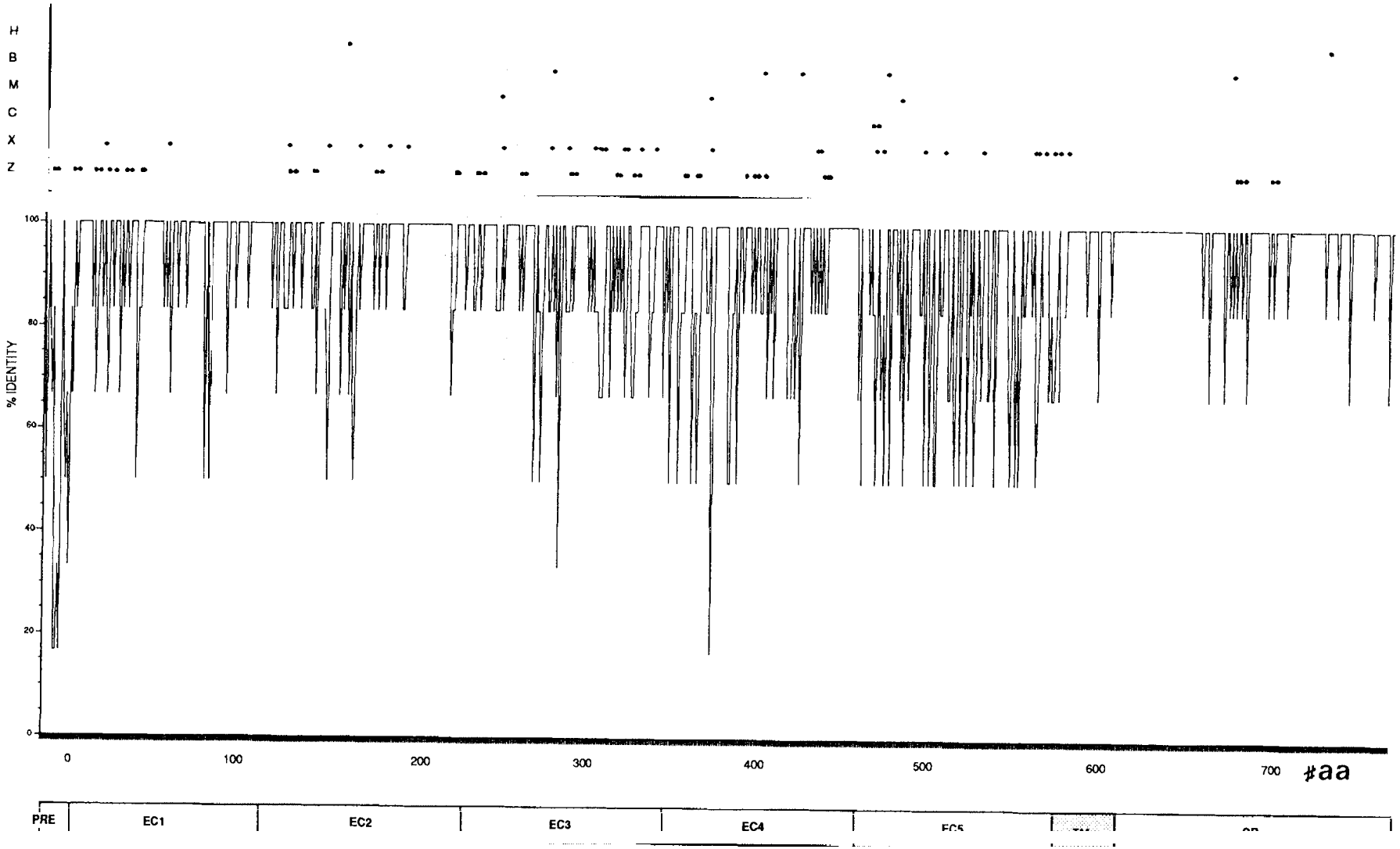

Fig. 3. Comparison of amino acid sequence of N-cadherins of different species. Six N-cadherin sequences (human [H], Walsh *et al.*, 1990; bovine [B], Liaw et al., 1990; mouse [M], Miyatani et al., 1989; chicken [C], Hatta et al., 1987; *Xenopus* [X], Detrick *et* al., 1990; Ginsberg et al., 1991; and zebrafish [Z], ZN-cadherin) were aligned using the "pileup" multisequence alignment program (Devereux et al., 1984). The **lower panel** is a graphic presentation of the calculated percent identity at each position. In the **upper panel,** the positions in which single amino acid deviations from the consensus were noted are marked, for each species. The scheme at the bottom depicts the cadherin protein divided into its major domains.

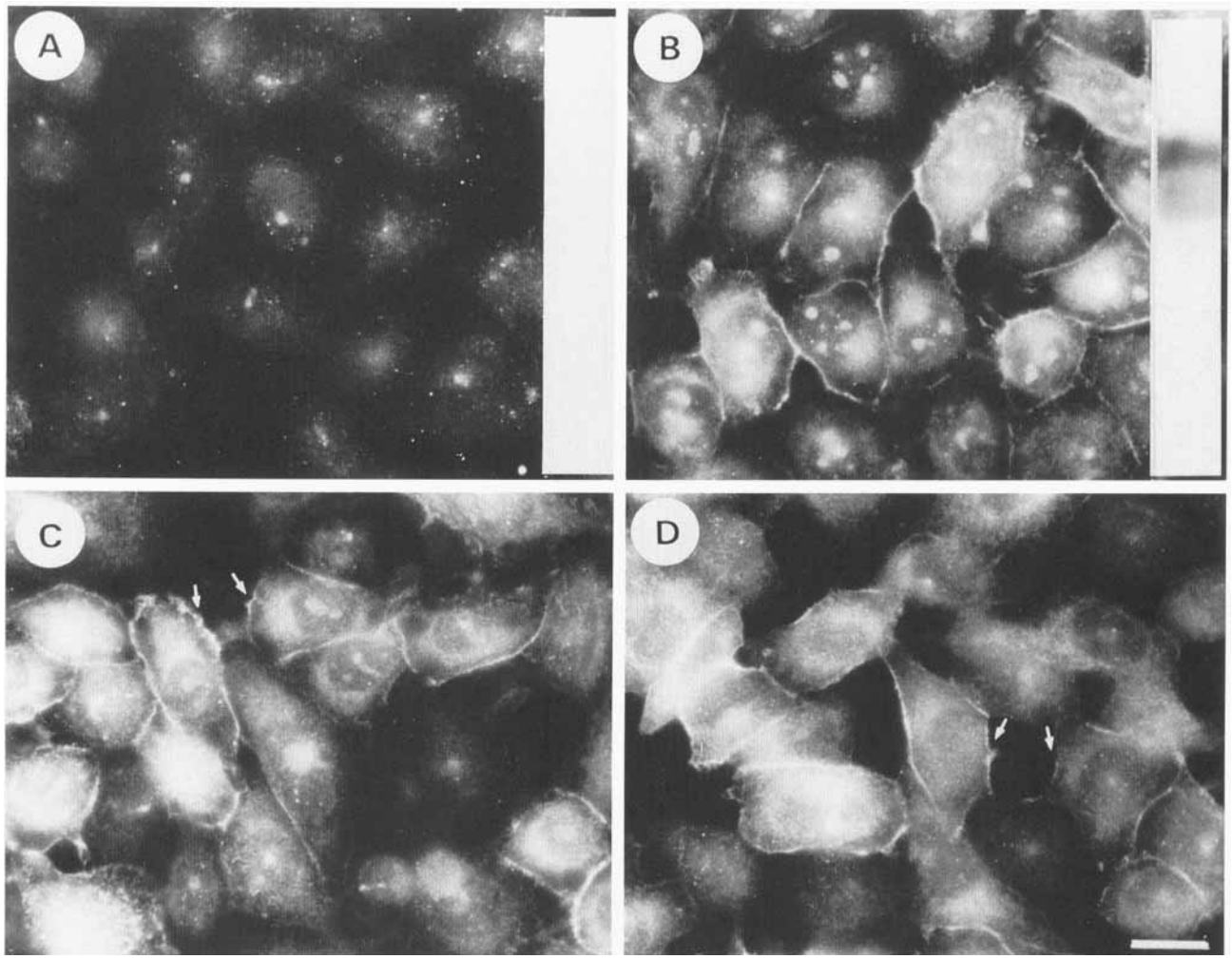

Fig. 4. Immunofluorescence staining, and Western blot analysis with R-851 anti-ZN-cadherin antibodies, of CHO cells (**A**), and CHO cells, stably transfected with ZN-cadherin cDNA (**B**). Note the immunoreactive 116 × 10^3 M_r band in the transfectants (**B**, arrowhead in **inset**) as compared to the lack of immunoreaction in non-transfected CHO cells (**inset** in A). To test the Ca^{2+}-dependence of the cell-cell junctions in the ZN-cadherin-transfectants, cells were incubated with 2 mM EGTA for 2 min (**C**) or 10 min (**D**). Note a progressive detachment of cells exposing the cadherin on the free cell surface (paired arrows). The bar indicates 10 μm.

bands in these embryos: one comigrated with, and was expressed at the same time as ZN-cadherin, while the other had a higher molecular weight, and was detected already in pre-gastrulation embryos. Interestingly, the expressed ZN-cadherin was initially diffusely distributed throughout the cells in numerous granules and only later, when epiboly was nearly complete, the protein segregated into discrete areas of cell-cell adhesion (see Fig. 11A).

Northern blot analysis, carried out using, independently, two non-overlapping ZN-cadherin cDNA probes (marked as "probe 1" and "probe 2" in Fig. 1), revealed that both probes hybridize to a single band of about 6 kb in total RNA extracts from adult brain and eyes. This RNA band was also present (though at lower levels) in extracts from adult trunk (Fig. 6). Analysis of samples extracted from embryos at different developmental stages indicated that ZN-cadherin mRNA was not present in early embryos, up to and including 30% epiboly and its expression was apparent at the shield (gastrulation) stage, about 1 hr later. This mRNA was of the same size as the one detected in adult tissues (Fig. 6).

To determine the spatio-temporal expression of ZN-cadherin mRNA during development, we carried out whole-mount in-situ hybridization. These experiments were carried out with the two ZN-cadherin cDNA probes and yielded essentially identical results. In line with the Northern blot analysis, ZN-cadherin transcripts were first detected by in-situ hybridization, at gastrulation. At this stage, the deep cells at the marginal zone of the blastoderm involute at the germ ring, giving rise to the hypoblast. Subsequently, these cells undergo convergence movements forming the axis at

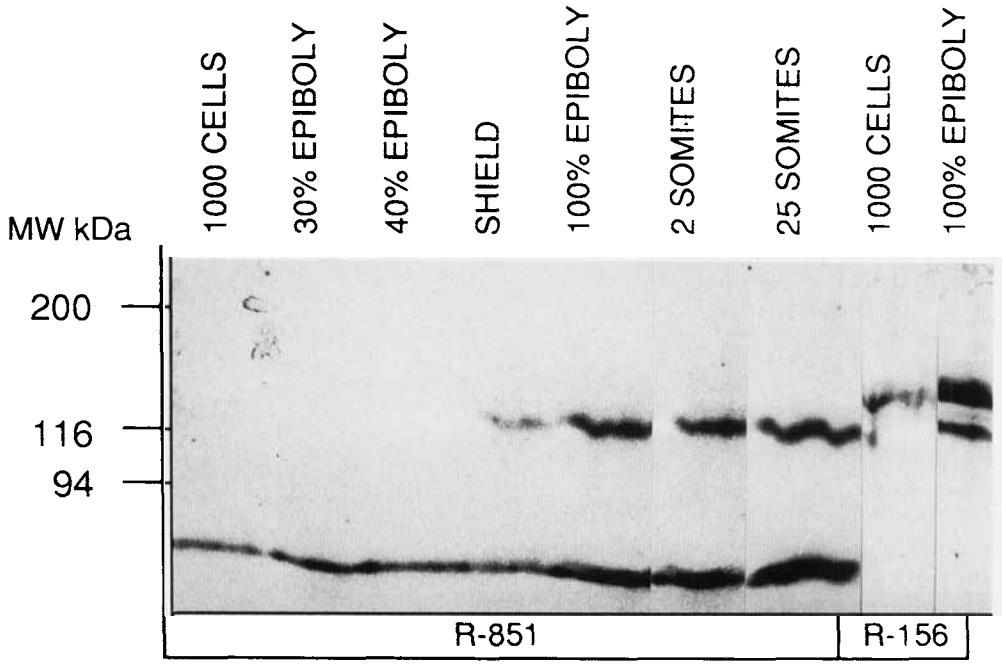

Fig. 5. Immunoblot analysis of protein extracts from zebrafish embryos at different developmental stages. Antibody R-851 detects a protein band of 116 × 10^3 M_r in embryos from shield stage and older. The pan-cadherin antibody R-156 detects an additional, earlier band of higher molecular weight. In each lane, 400 μg of protein was loaded, most of which consisted of yolk proteins.

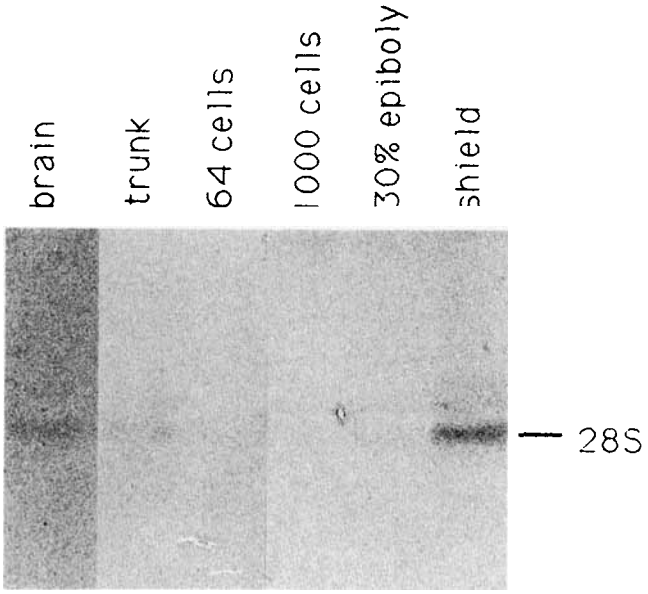

Fig. 6. Northern blot analysis of RNA from adult brain (including the eyes), adult trunk, as well as intact embryos at the 64 cell, 1,000 cell, 30% epiboly, and shield stages. The cDNA probe used here was probe 2 (identical results were obtained with probe 1; see Fig. 1). Total RNA (2.5–5 μg) was loaded in each lane. The position of 28S ribosomal RNA is marked.

the dorsal aspect of the gastrula (Warga and Kimmel, 1990). During this stage, the entire blastoderm appeared positive, with the germ-ring, the shield, and the embryonic axis showing a stronger signal (Fig. 7A and A′). Examination of cross sections indicated that both the epiblast and the hypoblast express ZN-cadherin (data not shown).

At 100% epiboly the strongly labeled region extended from the head to the yolk-plug region and beyond (Fig. 7B–B″). The notochord was visible as a more intensely labeled structure and in a dorsal view, positively labeled deep cells were detected in a defined zone, flanking the axis. Shortly later, at the tail-bud stage, ZN-cadherin-expressing regions lateral to the axis became narrower, and their borders sharply defined due to axial convergence (Fig. 7C–C″). The external enveloping layer (EVL) was apparently negative throughout development while the posterior part of the notochord and the mesoderm surrounding it expressed ZN-cadherin mRNA. A high level of expression was also detected in the head.

At three somites stage, ZN-cadherin was expressed in a variety of mesodermal structures, including the lateral mesoderm, the segmental plate, the somites, and the notochord as well as the optic placodes (Fig. 7D–D″). As segmentation proceeds, the embryo progressively elongates and somites are added at a rate of about 2 per hour. Unlike other vertebrates, in the zebrafish most of the somite becomes muscle, and only a small number of cells belong to the sclerotome or dermatome. In-situ hybridization of embryos at these stages indicated that up to about 16 hpf (15 somites) ZN-cadherin mRNA was detected in the segmental

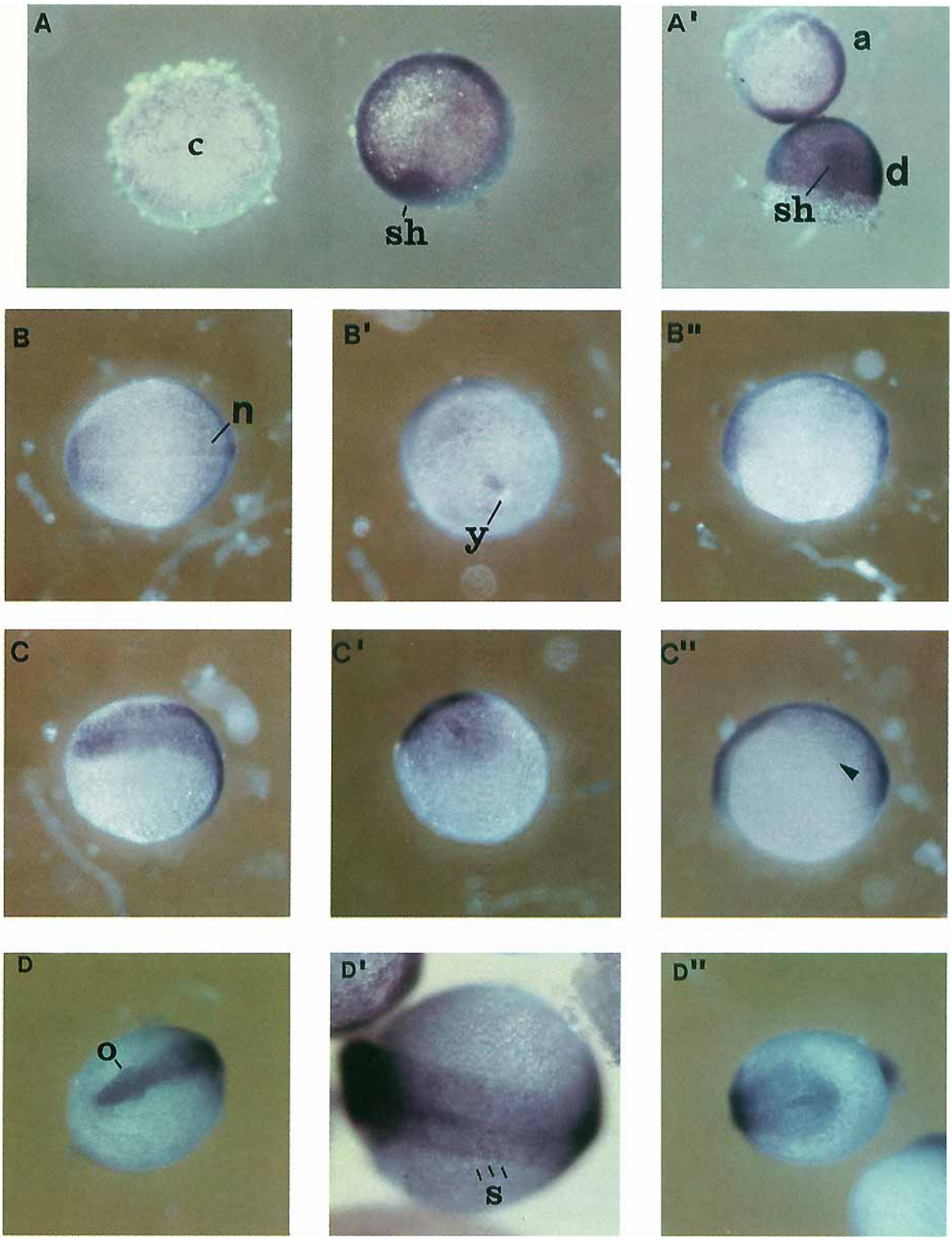

Fig. 7. Expression of ZN-cadherin mRNA in zebrafish embryos (5–15 hpf), as visualized by chromogenic whole-mount in-situ hybridization to zebrafish embryos: **A, A′:** Shield stage (5 hpf). Embryos are viewed from the animal pole (a) or from the dorsal aspect (d). The control embryo on the left (c) was hybridized with an irrelevant DNA. Note that the signal present in the shield (sh), is significantly higher than that detected elsewhere in the blastoderm. **B, B′, B″:** 100% epiboly stage. B: Dorsal view showing positive signal along the notochord (n); B′: Vegetal view showing the yolk-plug (y). The caudal aspect of the notochord appears labeled; B″: Lateral view showing the positive embryonic axis and deep cells. **C, C′, C″:** Tail-bud stage. C: Dorsal view showing the head and anterior part of the trunk. C′: The tail region, with positive notochord and mesoderm. The EVL appears negative; C″: Lateral view depicting the border between the positive deep cells, and negative EVL (arrow). **D, D′, D″:** Three somites stage embryos. D: The brain anlage and optic placodes (o) appear strongly labeled; D′: The trunk region with positive somites (s); D″: The tail region. Embryos (B–D) are presented with their anterior towards the left.

plate and in the epithelial somites (Fig. 8B). Upon initiation of myogenesis, concomitantly with the elongation of the somitic cells, the level of ZN-cadherin mRNA decreased. This was already apparent at 16 hpf, and at 24 hpf (30 somites) only the 5–6 caudal somites and the residual segmental plate were still positive (Fig. 8D,E). Once all myotomes differentiated into muscle, ZN-cadherin mRNA was no longer detectable (Fig. 8F). It is noteworthy that positive immunostaining for ZN-cadherin was still detected several hours after the respective RNA disappeared from muscle cells.

At all these stages the neural keel, and later the neural tube, brain, developing eyes (optic cups, lens, and retina) and otic vesicles were highly positive (Fig. 8C,G,H).

Ectopic Expression of ZN-Cadherin and Its Effects on Zebrafish Development

To study the effect of ZN-cadherin expression on cell sorting and segregation, we injected ZN-cadherin mRNA into embryos (about 70 embryos were injected in each experiment) at 2–4 cell stages and followed their subsequent development. Embryos injected with Lac-Z mRNA were used as controls both to monitor the specificity of the effect, as well as to examine the distribution of the injected transcripts. The capability of the injected mRNA to direct ZN-cadherin synthesis was demonstrated by Western blot analysis using R-851 antibodies. This assay revealed an immunoreactive band of 116×10^3 M_r in embryos at the beginning of epiboly, long before the endogenous protein is expressed (Fig. 9A). Treated embryos (10 to 15 at each time point) were further examined by immunolabeling.

In preliminary experiments, we found that injection of large volumes of solution (> 5 nl) and/or high amounts of mRNA (> 10 ng) into embryos had a marked non-specific toxic effect, manifested by arrest of development and excessive cell death during epiboly. Following a careful titration we defined an optimal injection volume of 1–2 nl, and mRNA concentration of 1 ng ml^{-1} which did not lead to non-specific effects.

Examination of injected embryos pointed to a significant and reproducible 1–2 hr delay in the onset of epiboly. Moreover, epiboly proceeded at a slower rate and was non-uniform. Nevertheless, gross deformations were not observed at that stage, and about 90% of the embryos survived (Fig. 9B and C).

After 24 hr all control embryos proceeded with normal development, while 50–90% of the embryos injected with ZN-cadherin mRNA showed severe deformations, ranging from numerous cell aggregates under the periderm (Fig. 10C,E) to deformed, asymmetrical, or apparently split axes (Fig. 10B,D).

To check for the earliest apparent effects of this ectopic over-expression, we have visualized the deep cells in injected embryos during epiboly, using nuclear staining with DAPI. In control embryos, several layers of deep cells were revealed by DAPI staining covering the animal pole and distributed evenly, covering the yolk (Fig. 9E). In contrast, injection of ZN-cadherin mRNA resulted in uneven distribution and clustering of deep cells (Fig. 9D). The extent of aggregation was variable in different embryos.

In order to determine whether the formation of these aggregates is spatially correlated to the expression and organization of ZN-cadherin protein, we used the antibody R-851 for immunostaining. As described above, the endogenous ZN-cadherin was first detected at 50% epiboly, but its localization to cell-cell junctions was delayed until epiboly was essentially complete (Fig. 11A). In contrast, immunofluorescence staining of embryos injected with ZN-cadherin mRNA, revealed high levels of the protein and clear localization at cell-cell junctions in the deep cell aggregates, at 60% epiboly (Fig. 11). It should, nevertheless be pointed out that 60% epiboly in the injected embryos was reached roughly at the same time as 90–100% epiboly in controls.

DISCUSSION

In this study we have characterized the spatio-temporal expression and morphogenetic potential of N-cadherin in developing zebrafish embryos. As a first step, we have isolated cDNA clones encoding N-cadherin from an embryonic zebrafish library. Amino acid sequence comparison of the fish molecule with its homologues in other species indicates that N-cadherin existed as a distinct adhesion molecule since early vertebrates (Teleost) evolution. The existence of long stretches of nearly complete identity, between N-cadherins of all species, suggests that these highly conserved regions are of great functional importance. However, despite the high overall conservation, in a comparison of N-cadherins of different species, a distinct hierarchy was noted in the frequency of singular species-specific deviations from the "consensus" of conserved regions. The number of such changes in the zebrafish N-cadherin is relatively high (62.4%), compared to *Xenopus* and higher vertebrates. This may reflect the level of evolutionary distance between these species, and is generally in line with the phylogenetic analysis recently performed for the EC1 domain of all known cadherins (Pouliot, 1992).

Analysis of ZN-cadherin adhesive properties was conducted both in transfected cultured cells and in RNA-injected embryos. The incorporation of the protein into cell-cell junctions following transfection confirmed that despite the phylogenetic gap, the zebrafish molecule can function in the mammalian cell environment, and interact with accessory proteins, such as catenins, which are known to be essential for cadherin function (Hirano et al., 1992; Takeichi et al., 1992). This result also indicates that the cloned cDNA encodes a fully functional molecule despite the fact that it contains a short presequence (only 35 amino acids) compared to those found in other species (100–150 amino acids). The first ATG found in the clone is not

within a Kozak consensus sequence (Kozak, 1984), suggesting that this is not the initiation ATG of the protein. It, nevertheless, functions as an effective signal peptide, probably due to the presence of the correct cleavage site required to yield the mature protein. This cleavage was previously shown to be essential for the function of uvomorulin (E-cadherin) (Ozawa and Kemler, 1990).

The in-vivo effect of over-expressing ZN-cadherin in early zebrafish embryos was studied by mRNA microinjection which resulted in a consistent delay in epiboly and an apparent microaggregation of deep cells. Consequently, the deep cells did not reach their axial destination, leading to missing or deformed organs in the 24-hr embryos. Two distinct mechanisms may be responsible for these effects: Microaggregation of the deep cells during epiboly might be the direct consequence of the ectopic over-expression of ZN-cadherin protein. Alternatively, cell aggregation might be attributed to the endogenous N-cadherin which is expressed according to the original time table, but due to the delay in the onset of epiboly this expression is premature relative to cell positioning. However, regardless of whether the cadherin directly responsible for the effect is the exogenous or the endogenous one, the results presented here suggest that premature expression of this molecule in deep cells, during convergence, severely affects further axial migration.

This is in line with a previous report on N-cadherin misexpression in *Xenopus* embryos. In the frog, overexpression of the protein mainly affected the neural tube and prevented cell mixing in the ectoderm during gastrulation (Detrick et al., 1990; Fujimori et al., 1990). In zebrafish, on the other hand, microaggregation of deep cells occurred not only at the region destined to become ectoderm, but at all parts of the gastrula.

An insight into the putative physiological roles of N-cadherin during zebrafish development was obtained by studying the expression and distribution of the respective mRNA and protein at different embryonic stages. ZN-cadherin mRNA was first detectable during gastrulation, indicating that it is zygotically transcribed and is not of maternal origin. This is consistent with previous findings in *Xenopus,* where N-cadherin was first detected at the neurula stage (in contrast with EP-cadherin which is maternally encoded) (Ginsberg et al., 1991). Analysis of protein expression using the R-851 anti ZN-cadherin antibody suggested that the protein is translated concomitantly with the appearance of its mRNA. It is noteworthy that the pan-cadherin antibody reacted with this protein, as well as with an additional maternal band, the nature of which is still unclear.

Whole-mount in situ hybridization analysis of ZN-cadherin mRNA in older embryos revealed its presence in the deep cells. As gastrulation proceeded, ZN-cadherin expression was detected both in the cells forming the axis, and in the deep cells approaching it. However, the hybridization signal was significantly stronger along the axis, where the cells form tight associations with each other. At these stages the deep cells undergo several types of morphogenetic movements, including convergence and extension, leading to the formation and elongation of the developing axis (Kimmel et al., 1990; Warga and Kimmel, 1990; Ho, 1992).

The localization of ZN-cadherin protein in the embryos indicated that its expression largely correlates with the assembly of migrating deep cells into the various axial epithelia, in line with previous reports on other species (Duband et al., 1988; Detrick et al., 1990). It was, however, noted that the expression of cadherin mRNA and protein per se is not sufficient for cell adhesion. Thus, it was shown here that while ZN-cadherin was already present in essentially all deep cells of gastrulating embryos, it became associated with cell-cell adhesions only later, towards the end of epiboly and only in cells present along the axis and flanking it. This is consistent with previous data on uvomorulin, which is present throughout early mouse embryogenesis, yet becomes functional only at the compaction stage when it is redistributed into cell-cell contacts (Vestweber et al., 1987). *Xenopus* U-cadherin is also present in cells during gastrulation but is not affecting their adhesive properties (Schneider et al., 1993). It is conceivable that additional factors are involved in cadherin function, such as the expression and state of phosphorylation of catenins (Matsuyoshi et al., 1992; Schneider et al., 1993).

As organogenesis begins, epithelial structures start to form and rearrange. This is accompanied by alterations in the expression of ZN-cadherin mRNA, which is prominent in the brain, neural keel, optic vesicles, otic placodes and vesicles, and in the epithelial somites. This increase is, often, transient, and when some of these epithelial structures change, the level of ZN-cadherin mRNA sharply declines. This is especially apparent in the developing somites, in which ZN-cadherin (RNA as well as protein) are down regulated concomitantly with muscle differentiation. A similar phenomenon was previously reported for chicken (Hatta et al., 1987; Duband et al., 1988).

In conclusion, the results presented here point to the importance of tightly regulated expression and subcellular localization of N-cadherin for normal cell positioning and morphogenesis during development. This control is highly specific both in space and time, allowing cells to freely migrate to their destination and then to assemble into epithelia, or to lose epithelial organization.

EXPERIMENTAL PROCEDURES

Animals and Embryos

Zebrafish were raised essentially as described by Westerfield (1989). Adult fish were maintained in charcoal-filtered, double distilled water, supplemented with salts (Eaton and Farley, 1974). Embryos were obtained over marbles as described (Westerfield, 1989).

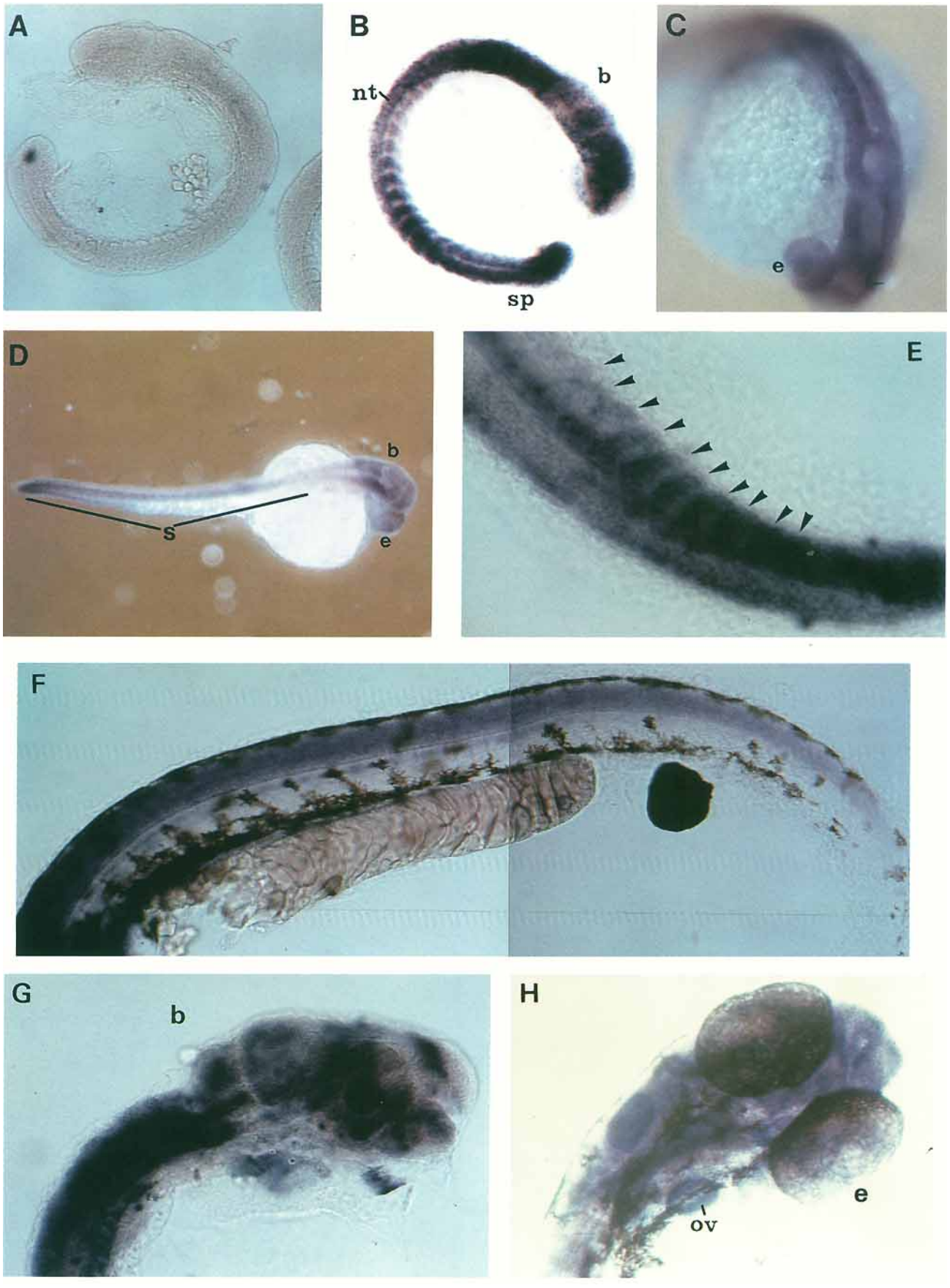

Fig. 8.

After cleaning and staging embryos were incubated in Petri dishes in "Neviot" mineral water (Neviot, Israel) at 28°C on a thermostated stage. Development was monitored microscopically.

Cloning and Sequencing of ZN-Cadherin

A λgt10 c-DNA library of 0–48 hr zebrafish embryos (kindly supplied by A. Molven, Haukeland Hospital, Bergen, Norway) was screened under low stringency conditions (Sambrook et al., 1989) with a mixture of *Xenopus* EP- and N-cadherin probes (Ginsberg et al., 1991). Positive clones were subsequently re-screened with the *Xenopus* N-cadherin cDNA at a high stringency. Isolated cDNA clones were subcloned into the *EcoRI* site of the Bluescript plasmid (Stratagene, La-Jolla, CA), and sequenced as double-stranded templates, using the Sequenase kit (U.S. Biochemicals, Cleveland, OH). Computer sequence analyses were performed using the GCG7 software package (Devereux et al., 1984). The full-length cDNA was inserted into the pECE eukaryotic expression vector (Ellis et al., 1986) in the *SalI* site and the resulting plasmid denoted ZN-pECE.

Preparation of Antibodies to ZN-Cadherin

To prepare antibodies to ZN-cadherin, we expressed in bacteria a segment of the molecule corresponding to amino acids 370–572 of the extracellular domain of the mature protein. This region shows the highest diversity between different cadherins (Hatta et al., 1988). PCR primers were prepared, providing at their 5′ end an extra sequence of AAACAT which, together with an intrinsic ATG, created an *NdeI* site. The PCR fragment was blunted, cut with *NdeI,* and subcloned into the pET3a bacterial expression vector (Studier et al., 1990). The 203 amino acids peptide was expressed in BL21(DE3) bacteria following IPTG induction (1 mM) and the bacterial inclusion bodies purified as described by Sambrook et al. (1989). The expressed cadherin fragment (comprising about 70% of the total bacterial proteins) was emulsified in complete Freund's adjuvant (Difco, Detroit, MI) and injected in 2.5–5 mg proteins to rabbits. Following two booster injections at 2-week intervals, the animals were bled, and the resulting serum (R-851) tested by immunofluorescence staining and Western blot of transfected cells (see below). Antibodies were affinity purified on the peptide used for injection, bound to a p-nitrophenolchloroformate-activated Sepharose (Wilchek and Miron, 1982). The eluted antibody was used at 30 μg ml^{-1} for immunofluorescence, and at 5 μg ml^{-1} for Western blot analysis.

Extraction of Proteins, SDS-PAGE, and Immunoblots

Cultured cells and adult zebrafish tissues were extracted in Laemmli sample buffer (Laemmli, 1970). Embryos were extracted in RIPA buffer containing protease inhibitors (Resnitzky et al., 1992). For 20 to 100 embryos 50 to 100 μl of RIPA were used, and the embryos were passed through a 23 gauge syringe needle. The extract was centrifuged for 10 min at 4°C, and the supernatant kept in −70°C. The extracts (200–400 μg of protein) were electrophoresed on 8% polyacrylamide slab gels. Polypeptides were electroblotted onto Hybond-C membranes (Amersham, UK) and immunolabeled using anti-ZN-cadherin antibody R-851 or pan-cadherin serum R-156 (Geiger et al., 1990). Detection of immunoreactive bands was performed either by ^{125}I-labeled- or alkaline phosphatase-conjugated secondary antibodies, or protein A.

Cell Culture and Transfection

CHO cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), at 37°C in a humidified atmosphere of 7% CO_2 in air. The cells were co-transfected with the ZN-pECE plasmid and the pSV2-neo plasmid (Southern and Berg, 1982) using the calcium phosphate transfection procedure (Graham and Van Der Eb, 1973). Transfectants were selected using 600 μg ml^{-1} of G-418 (Geneticin, GIBCO, Grand Island, NY) and positive clones identified by immunoblot and immunofluorescence analyses using the anti-ZN-cadherin antibody.

Immunofluorescence Labeling of Cultured Cells

Cells were cultured on glass coverslips, fixed and permeabilized in methanol (−20°C, 7 min), followed by acetone (−20°C, 10 sec), and washed in PBS. Cells were labeled using affinity purified R-851 antibodies and rhodamine-conjugated goat anti rabbit antibodies (Jackson Labs, Bar Harbor, ME) as described (Volk and Geiger, 1984).

Whole-Mount Immunostaining of Embryos

Whole embryos were labeled as described by Westerfield (1989), except that the secondary antibodies were conjugated to rhodamine or to Cy-3 (Jackson Labs). All incubations with antibodies were for 16 hr at 4°C, and the washes were conducted at room temperature. Individual cells were visualized by nuclear staining with DAPI (4′,6′-diamidino-2-phenilindole, Sigma,

Fig. 8. Distribution of ZN-cadherin mRNA in embryos at 15–36 hpf: **A:** A 15 hpf embryo hybridized with an irrelevant DNA, used here as a negative control. **B:** 15 hpf embryo hybridized with ZN-cadherin cDNA. Note the positive labeling in the brain (b), neural tube (nt), somites, and segmental plate (sp). The anterior somites show a weaker signal. **C:** The head region of a 20 hpf embryo. Note the positive labeling of the brain and the eye (e) including the optic cup and the lens. **D:** A dorsal view of a 25 hpf embryo. Note the strong labeling of the caudal somites, compared to the negative labeling of the more rostral ones. The brain (b), eye (e), and neural tube remain strongly positive (s = somites). **E:** A detail of a 25 hpf embryo tail. The somites are labeled with arrowheads. Note the gradient of labeling from the segmental plate, through the caudal somites, to the negative, more rostral somites. **F:** A 36 hpf embryo. All somites are already negative, while the neural tube is positively labeled. **G:** Head of a 25 hpf embryo showing strong labeling of all parts of the brain (b) and the eye. **H:** Head of a 36 hpf embryo showing the positive otic vesicles (ov).

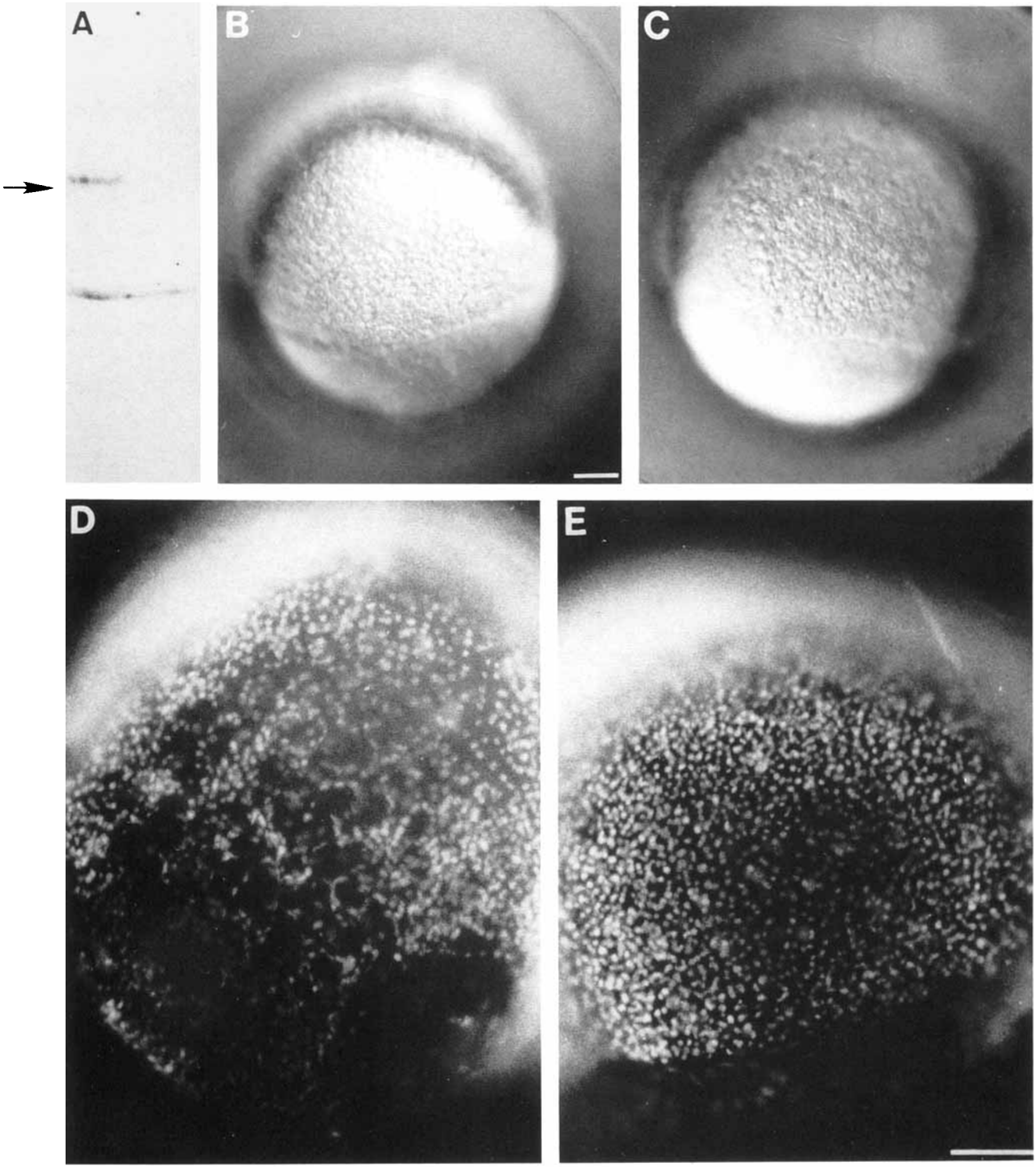

Fig. 9. Effect of ectopic ZN-cadherin expression on early zebrafish embryos. **A:** Western blot analysis with antibody R-851, showing, on the left, the 116 × 10^3 M_r band of ZN-cadherin (arrow), expressed at 30% epiboly in embryos injected with ZN-cadherin mRNA. On the right, the band is missing in uninjected embryos at the same developmental stage. **B, C:** Two live embryos at 60% epiboly, one injected with ZN-cadherin mRNA (B) and the other with Lac-Z mRNA (C) as photographed using Nomarski optics. Both embryos look normal. **D, E:** Two embryos at 60–70% epiboly, visualized by DAPI staining which reveals the localization of the nuclei. In the embryo injected with ZN-cadherin mRNA (D), the nuclei are clustered in microaggregates, while in the control embryo, injected with Lac-Z (E), the cells are evenly spread over the yolk. The bars in B and E represent 100 μm.

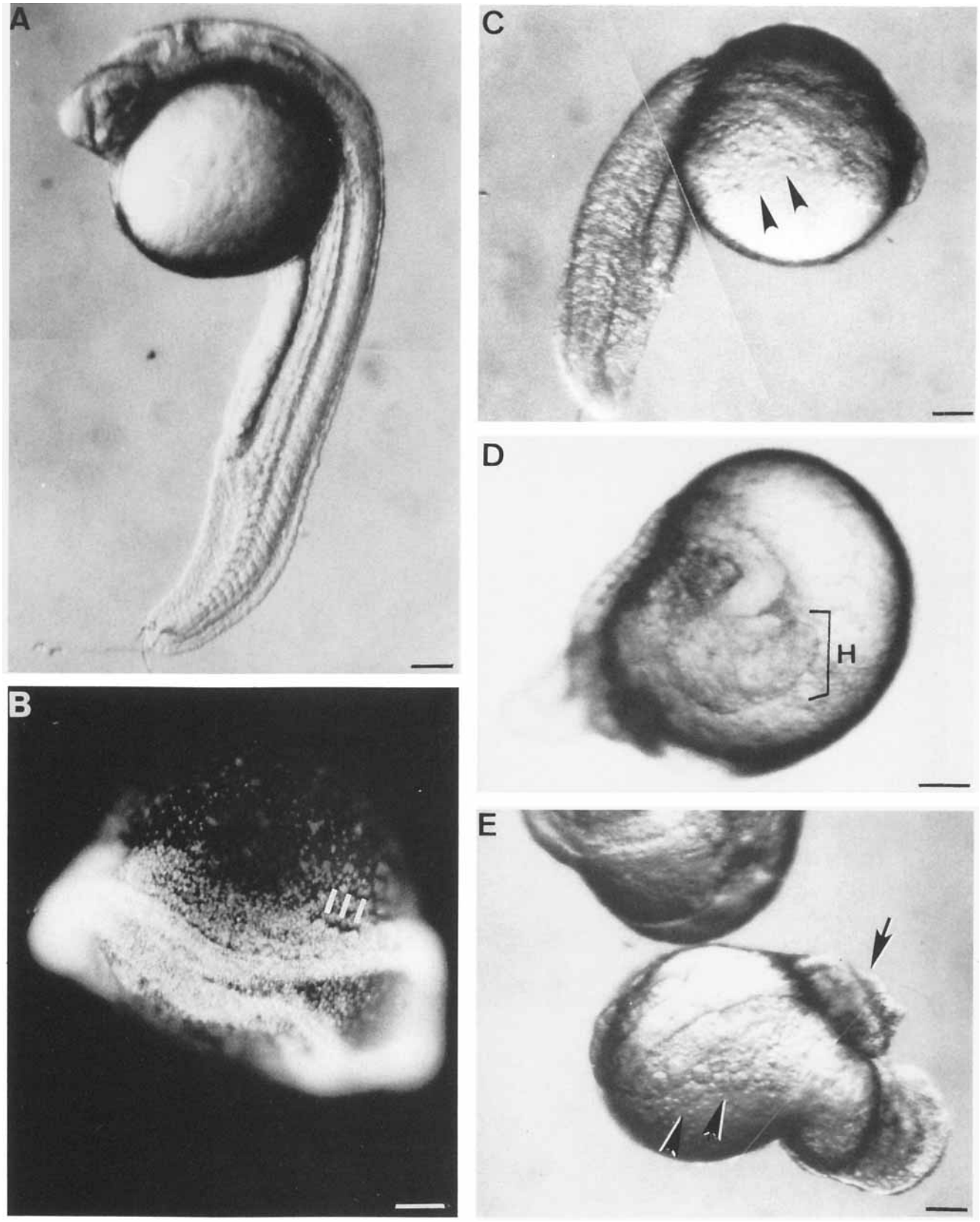

Fig. 10. Effect of ectopic ZN-cadherin expression after 24 hr of development. **A:** An embryo injected with Lac-Z mRNA, showing a normal phenotype. **B–E:** Embryos injected with ZN-cadherin mRNA, showing different types of deformations. In (B) a bipartite embryo is visualized by DAPI staining, showing the neural tube and the separated somites on one side (bars). In (C) the embryo has a shorter and thicker tail, and cell aggregates are visible over the yolk (arrowheads). (D) shows the head region (H) of another embryo, where the brain structure is completely distorted and the embryo's axis is crooked. In (E) the embryo has a "tumor" on its dorsal side (arrow), and many cell aggregates over the yolk (arrowheads). The scale bar in each photograph represents 100 μm.

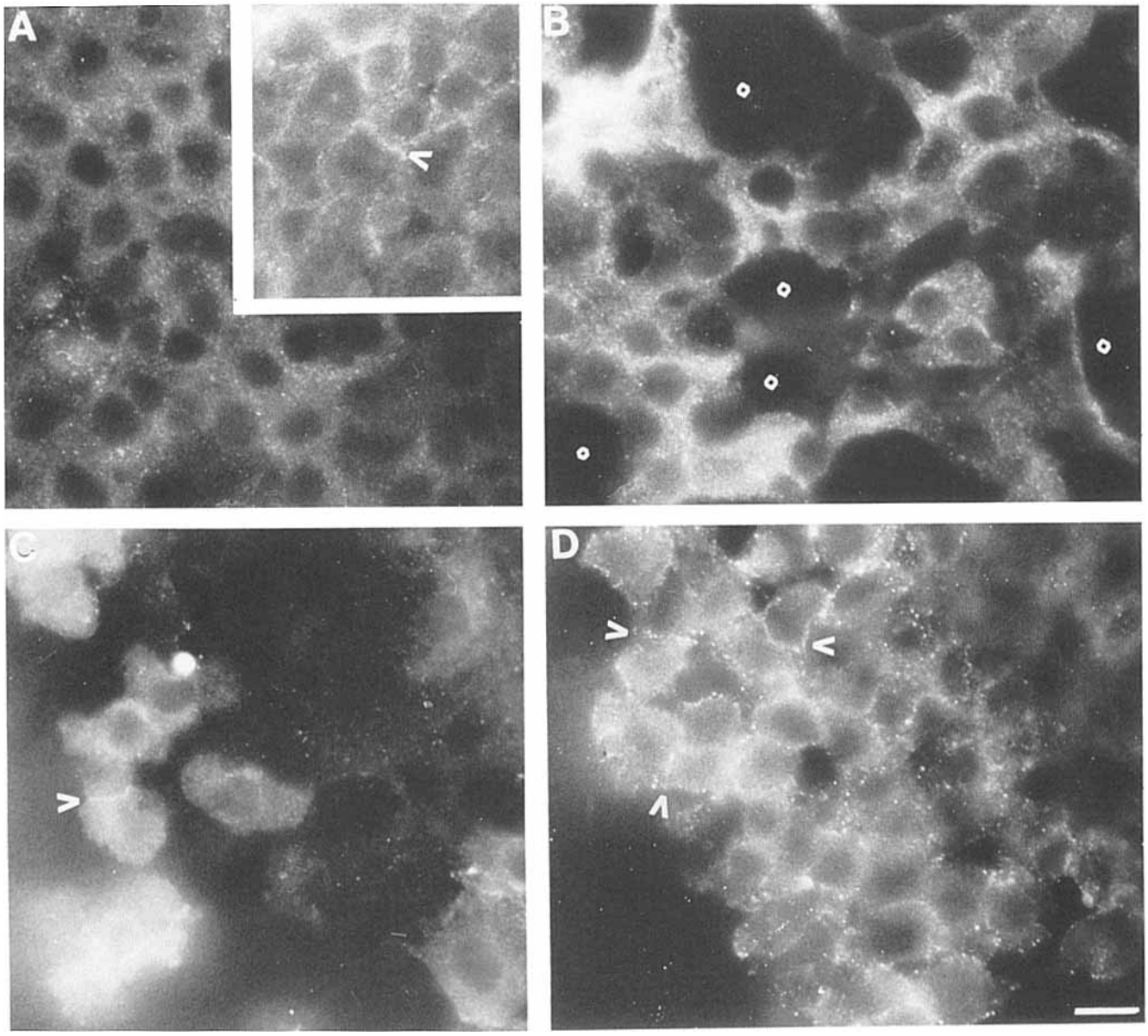

Fig. 11. Immunofluorescent analysis of ZN-cadherin protein distribution in embryos injected with ZN-cadherin mRNA, using antibody R-851. **A:** Uninjected embryos at 60% (A) and 100% epiboly (**inset**), showing the late organization of the endogenous ZN-cadherin protein. Note that at 60% epiboly the staining is granular and the cells are evenly spread, while in 100% epiboly the protein is localized in intercellular boundaries (arrowhead, also in C,D), and the cells are well organized. **B,C,D:** Aggregates formed in different ZN-cadherin injected embryos at 60–70% epiboly. Note the variability in the levels of protein expression and organization, as well as in the extent of aggregation. In (B) the cells are tightly aggregated, leaving empty gaps between the aggregates (diamonds). In (C) a few small aggregates are seen, isolated from each other, and ZN-cadherin protein is organized in cell-cell junctions. In (D) a larger aggregate is shown, expressing the protein which again is localized to junctions. The bar represents 100 μm.

St. Louis, MO) at 2.5 μg ml^{-1} for 30 min. Stained embryos were mounted in elvanol (Mowiol 48-8, Hoechst, Germany) between two spaced coverslips.

Whole-Mount In Situ Hybridization

Fixation, permeabilization and proteinase K digestion of embryos at different developmental stages were performed according to Puschel et al. (1992). Hybridization was performed according to Krauss et al. (1991) except that pre-hybridization was carried out for 16–24 hr. The cDNA probes were labeled with Boehringer random priming kit, using digoxigenin (DIG) 11-dUTP (Boehringer, Mannheim, Germany) according to manufacturer's protocol and the reaction was carried out for 16 hr at 14°C, followed by the addition of Klenow enzyme (5 U) and incubation for an additional 4 hr at

room temperature. As a negative control we have used the λ-phage DNA supplied with the random priming kit. The probes were checked in dot blot, and used at 1:100 dilution. Alkaline phosphatase-conjugated anti-DIG antibodies (Boehringer) were diluted 1:1,000, preadsorbed on non hybridized embryos for at least 1 hr before incubation, and used at a final dilution of 1:2,000.

RNA Purification and Northern Blot Analysis

RNA was extracted from adult fish tissues using the LiCl-Urea procedure (LeMeur et al., 1981). Brain and eyes were extracted together, and the rest of the body was extracted separately. RNA extraction from small numbers of embryos was carried out in microfuge tubes. Total RNA (5 to 7.5 μg) was electrophoresed in agarose-formaldehyde gels (Sambrook et al., 1989). The RNA was blotted onto Magna Nt nylon membranes (MSI, Westboro, MA), stained with methylene blue and subjected to hybridization under high-stringency conditions. rRNA visualized by methylene blue was used as a size marker and for standardization of RNA ammount. cDNA probes (^{32}P-labeled) were prepared using a random-priming DNA labeling kit (Boehringer).

In Vitro Transcription

Capped RNA transcripts encoding the full length ZN-cadherin were transcribed in vitro, using, as a template, the insert in a Bluescript vector linearized at the KspI site. Control mRNA was transcribed from the pBSFZ7 lac-Z vector kindly provided to us by Jeremy Wegner (Eugene, Oregon). Transcription was performed as described by Krieg and Melton (1984) using T3 RNA polymerase with the Riboprobe kit (Promega, Madison, WI). RNA transcripts were treated with RNase-free DNaseI, extracted with phenol-chloroform, and precipitated in the presence of NH_4Ac. The RNA was resuspended in DEPC treated water, containing 0.05% phenol-red.

Microinjection of RNA Into Embryos

Chorionated embryos at two to four cells stage were microinjected in an agarose mold with under a dissection microscope. Glass capillaries (1 mm fiber-filled; WPI, Sarasota, FL) were pulled in a horizontal puller (Sutter Instruments, San Rafael, CA), and mounted on a Leitz micromanipulator (Germany), connected to a semi-automatic microinjector (Eppendorf 5242). The RNA was injected into the interface between the cells and the yolk, and the injection volume was 1–2 nl containing 1–2 ng RNA. Injected embryos were maintained at 28°C and photographed under the dissection microscope using a Reichert photo-automatic camera (Austria).

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REFERENCES

Albelda, S.M., and Buck, C.A. (1990) Integrins and other cell adhesion molecules. FASEB J 4:2868–80.

Cunningham, B.A., and Edelman, G.E. (1990) Structure, expression, and cell surface modulation of cell adhesion molecules. In: "Morphoregulatory Molecules," Edelman, G.M., Cunningham, B.A., and Thiery, J.P. (eds). New York: John Wiley and Sons, pp 9–40.

Detrick, R.J., Dickey, D., and Kintner, C.R. (1990) The effects of N-cadherin misexpression on morphogenesis in Xenopus embryos. Neuron 4:493–506.

Devereux, J., Haeberli, P., and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12:387–395.

Duband, J.L., Volberg, T., Sabanay, I., Thiery, J.P., and Geiger, B. (1988) Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. Development 103:325–344.

Eaton, R.C., and Farley, R.D. (1974) Growth and reduction of depensation of zebrafish, *Brachidanio rerio,* reared in the laboratory. Copeia 1:204–209.

Edelman, G.R. (1993) A golden age for adhesion. Cell Adhesion Comm. 1:1–7.

Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A., and Rutter, W.J. (1986) Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. Cell 45:721–732.

Fujimori, T., and Takeichi, M. (1993) Disruption of epithelial cell-cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin. Mol. Biol. Cell 4:37–47.

Fujimori, T., Miyatani, S., and Takeichi, M. (1990) Ectopic expression of N-cadherin perturbs histogenesis in Xenopus embryos. Development 110:97–104.

Geiger, B. (1989) Cytoskeleton-associated cell contacts. Curr. Opin. Cell Biol. 1:103–109.

Geiger, B., and Ayalon, O. (1992) Cadherins. Ann. Rev. Cell Biol. 8:307–332.

Geiger, B., and Ginsberg, D. (1991) The cytoplasmic domain of adherens-type junctions. Cell Motil. Cytoskeleton 20:1–6.

Geiger, B., Volberg, T., Ginsberg, D., Bitzur, S., Sabanay, I., and Hynes, R.O. (1990) Broad spectrum pan-cadherin antibodies, reactive with the C-terminal 24 amino acid residues of N-cadherin. J. Cell Sci. 97:4.

Ginsberg, D., DeSimone, D., and Geiger, B. (1991) Expression of a novel cadherin (EP-cadherin) in unfertilized eggs and early Xenopus embryos. Development 111:315–325.

Graham, F.L., and Van Der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467.

Hatta, K., and Takeichi, M. (1986) Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature (London) 320:447–449.

Hatta, K., Okada, T.S., and Takeichi, M. (1985) A monoclonal antibody disrupting calcium-dependent cell-cell adhesion of brain tissues: Possible role of its target antigen in animal pattern formation. Proc. Natl. Acad. Sci. U.S.A. 82:2789–2793.

Hatta, K., Takagi, S., Fujisawa, H., and Takeichi, M. (1987) Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev. Biol 120:215–227.

Hatta, K., Nose, A., Nagafuchi, A., and Takeichi, M. (1988) Cloning and expression of cDNA encoding a neural calcium-dependent cell

adhesion molecule: Its identity in the cadherin gene family. J Cell Biol 106:873–81.

Heasman, J., Ginsberg, D., Geiger, B., Goldstone, K., Pratt, T., Yoshido-Noro, C., and Wylie, C. (1994) A functional test for maternally inherited cadherin in Xenopus shows its importance in cell adhesion at the blastula stage. Development 120:49–57.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S., and Takeichi, M. (1992) Identification of a neural α-catenin as a key regulator of cadherin function and multy cellular organization. Cell 70:293–301.

Ho, R.K. (1992) Cell movements and cell fate during zebrafish gastrulation. Development (Suppl):65–73.

Kemler, R. (1993) From cadherins to catenins: Cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet. 9:317–321.

Kimmel, C.B. (1989) Genetics and early development of zebrafish. Trends Genet. 5:283–288.

Kimmel, C.B., Warga, R.M., and Schilling, T.F. (1990) Origin and organization of the zebrafish fate map. Development 108:581–594.

Kozak, M. (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNA. Nucleic Acids Res. 12:857–872.

Krauss, S., Johansen, T., Korzh, V., and Fjose, A. (1991) Expression of the zebrafish paired box gene pax[zf-b] during early neurogenesis. Development 113:1193–1206.

Krieg, P.A., and Melton, D.A. (1984) Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. 12:7057–7070.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

LeMeur, M., Glanville, N., Mandel, J.L., Gerlinger, P., Palmiter, R., and Chambon, P. (1981) The ovalbumin gene family: Hormonal control of X and Y gene transcription and mRNA accumulation. Cell 23:561–571.

Liaw, C.W., Cannon, C., Power, M.D., Kiboneka, P.K., and Rubin, L.L. (1990) Identification and cloning of two species of cadherins in bovine endothelial cells. EMBO J. 9:2701–2708.

Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S., and Takeichi, M. (1992) Cadherin mediated cell-cell adhesion is perturbed by v-src thyrosine phosphorylation in metastatic fibroblasts. J. Cell Biol. 118:703–714.

Matsuzaki, F., Mege, R.M., Jaffe, S.H., Friedlander, D.R., Gallin, W.J., Goldberg, J.I., Cunningham, B.A., and Edelman, G.M. (1990) cDNAs of cell adhesion molecules of different specificity induce changes in cell shape and border formation in cultured S180 cells. J. Cell Biol. 110:1239–1252.

Miyatani, S., Shimamura, K., Hatta, M., Nagafuchi, A., Nose, A., Matsunaga, M., Hatta, K., and Takeichi, M. (1989) Neural cadherin: Role in selective cell-cell adhesion. Science 245:631–635.

Mullins, M.C., and Nusslein-Volhard, C. (1993) Mutational approaches to studying embryonic pattern formation in the zebrafish. Curr. Opin. Genet. Dev. 3:648–654.

Nagafuchi, A., and Takeichi, M. (1988) Cell binding function of E-cadherin is regulated by the cytoplasmic domain. Embo J. 7:3679–3684.

Nose, A., Nagafuchi, A., and Takeichi, M. (1988) Expressed recombinant cadherins mediate cell sorting in model systems. Cell 54:993–1001.

Ozawa, M., and Kemler, R. (1990) Correct proteolytic cleavage is required for the cell adhesive function of uvomorulin. J. Cell Biol. 111:1645–1650.

Ozawa, M., Baribault, H., and Kemler, R. (1989) The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. EMBO J. 8:1711–1717.

Ozawa, M., Hoschutzky, H., Herrenknecht, K., and Kemler, R. (1990a) A possible new adhesive site in the cell-adhesion molecule uvomorulin. Mech. Dev. 33:49–56.

Ozawa, M., Ringwald, M., and Kemler, R. (1990b) Uvomorulin-catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule. Proc. Natl. Acad. Sci. U.S.A. 87:4246–4250.

Pouliot, Y. (1992) Phylogenetic analysis of the cadherin superfamily. Bioessays 14:743–748.

Puschel, A.W., Gruss, P., and Westerfield, M. (1992) Sequence and expression pattern of pax-6 are highly conserved between zebrafish and mice. Development 115:643–651.

Resnitzky, D., Tiefenbrun, N., Berrisi, H., and Kimchi, A. (1992) Interferon and interleukin 6 suppress phosphorylation of the retinoblastoma protein in growth-sensitive hematopoietoc cells. Proc. Natl. Acad. Sci. 89:402–406.

Ringwald, M., Schuh, R., Vestweber, D., Eistetter, H., Lottspeich, F., Engel, J., Dolz, R., Jahnig, F., Epplen, J., et al. (1987) The structure of cell adhesion molecule uvomorulin. Insights into the molecular mechanism of $Ca2^{+}$-dependent cell adhesion. EMBO J 6:3647–3653.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) "Molecular Cloning. A Laboratory Manual." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schneider, S., Herrenknecht, K., Butz, S., Kemler, R., and Hausen, P. (1993) Catenins in Xenopus embryogenesis and their relation to the cadherin-mediated cell-cell adhesion system. Development 118: 629–640.

Southern, P.J., and Berg, P. (1982) Transformation of mamalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Gene. 341:327–341.

Studier, W.F., Rosenberg, H.R., Dunn, J.J., and Dubendroff, J.W. (1990) Use of T7 RNA polimerase to direct expression of cloned genes. Methods Enzymol. 185:60–89.

Takeichi, M. (1990) Cadherins: A molecular family important in selective cell-cell adhesion. Annu. Rev. Biochem. 59:237–252.

Takeichi, M., Hirano, S., Matsuyoshi, N., and Fujimori, T. (1992) Cytoplasmic control of cadherin-mediated cell-cell adhesion. Cold Spring Harbor Symp. Quant. Biol. 57:327–334.

Vestweber, D., Gossler, A., Boller, K., and Kemler, R. (1987) Expression and distribution of cell adhesion molecule uvomorulin in mouse preimplantation embryos. Dev. Biol. 124:451–456.

Volk, T., and Geiger, B. (1984) A 135-kd membrane protein of intercellular adherens junctions. EMBO J. 3:2249–2260.

Volk, T., and Geiger, B. (1986) A-CAM: A 135-kD receptor of intercellular adherens junctions. II. Antibody-mediated modulation of junction formation. J. Cell Biol. 103:1451–1464.

Walsh, F.S., Barton, C.H., Putt, W., Moore, S.E., Kelsell, D., Spurr, N., and Goodfellow, P.N. (1990) N-cadherin gene maps to human chromosome 18 and is not linked to the E-cadherin gene. J. Neurochem. 55:805–812.

Warga, R.M., and Kimmel, C.B. (1990) Cell movements during epiboly and gastrulation in zebrafish. Development 108:569–580.

Westerfield, M. (1989) "The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (*Brachidanio rerio*)". Eugene, OR: University of Oregon Press.

Westerfield, M., Wagner, J., Jegalian, B.G., DeRobertis, E.M., and Puschel, A.W. (1992) Specific activation of mammalian *Hox* promoters in mosaic transgenic zebrafish. Genes Dev. 6:591–598.

Wilchek, M., and Miron, T. (1982) Immobilization of enzyme and affinity ligands onto agarose via stable and uncharged carbamate linkages. Biochem. Int. 4:629–635.