Characterization of genes expressed during mesoderm formation and anteroposterior patterning in carp (*Cyprinus carpio*)

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Characterization of genes expressed during mesoderm formation and anteroposterior patterning in carp (*Cyprinus carpio*)

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op gezag van de rector magnificus
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Stellingen

1 Het is aannemelijk dat het *carp cdx1* gen essentieel is voor de specificatie van positionele waarden in het caudale deel van het embryo, middels regulatie van *Hox* genexpressie.
Pownall et al. Development 122, 3881-3892 (1996); Subramanian et al. Cell 83, 641-653 (1995); dit proefschrift (hoofdstuk 2)

2 De investering in subtractieve hybridisatie betaalt zich bij het zoeken naar stadium-specifieke genen in cDNA banken royaal terug in het efficiënte screenen.
Dit proefschrift (hoofdstuk 4)

3 Het is aannemelijk dat het cth1 eiwit als translatiefactor werkt en niet als transcriptiefactor.
Dit proefschrift (hoofdstuk 5)

4 Verondersteld kan worden dat het *cth1* gen betrokken is bij het behoud van cellulair potentieel in blastomeren tijdens de klievingsfase en in mesendodermale cellen tijdens de gastrulatie.
Dit proefschrift (hoofdstuk 6)

5 Niet door zijn biologische eigenschappen maar door het mechanisme van vraag en aanbod op de wetenschappelijke markt, is de zebravis een "beter" model voor onderzoek naar de vertebraten-ontwikkeling dan de karper.

6 Northern Blot analyse is niet zonder meer geschikt om te bepalen of een maternale expressie al dan niet overgaat in een embryonale fase van expressie.
Ma et al. Oncogene 9, 3329-3334 (1994); dit proefschrift (hoofdstuk 5)

7 Het gebruik om de namen van humane Hox genen in hoofdletters weer te geven verleent de mens ten onrechte een hogere status dan andere diersoorten, en zou daarom afgeschaft moeten worden.
Bürglin (1994) A comprehensive classification of homeobox genes. In Duboule (ed); Guidebook to the homeobox genes.

8 Waar het de distributie van RNA betreft, zou eigenlijk gesproken moeten worden van een "transcriptiepatroon" in plaats van een "expressiepatroon".

9 Een beoordelingssysteem voor manuscripten waarin anonieme referenten de concurrenten zijn van de auteurs is gezien de mogelijke intriges de wetenschappelijke variant van "the Bold and the Beautiful".

10 Uit het oogpunt van verkeersveiligheid is het aan te bevelen om óók de minimumsnelheid op de snelwegen te verhogen naar 120 kilometer per uur.

11 Het is niet de beweging, maar de kwaliteit ervan, die bepaalt of ballet een kunst of een sport is.

Stellingen behorend bij het proefschrift "Characterization of genes expressed during mesoderm formation and anteroposterior patterning in carp (Cyprinus carpio)" van Carine J.M. Stevens, Wageningen, 3 oktober 1997

CONTENTS

Abbreviations

aa	amino acids
a.f.	after fertilization
a-p	anteroposterior
BMP	bone morphogenetic protein
bp	basepairs
cDNA	complementary DNA
C3H	cysteine-three-histidine
CNS	central nervous system
CRE	cth response element
cth	cysteine-three-histidine
DC	deep cells
DLT	dorsal longitudinal tract
EVL	enveloping layer
FGF	fibroblast growth factor
h	hours
ISH	in situ hybridization
MBT	midblastula transition
mRNA	messenger RNA
rRNA	ribosomal RNA
nt	nucleotides
PCR	polymerase chain reaction
p.f.	post fertilization
pfu	plaque forming units
r	rhombomere
r4	rhombomere 4
SEP	sperm entry point
TGFß	transforming growth factor ß
TIS	tristetraprolin induced sequence
TNF-α	tumor necrosis factor α
TTP	tristetraprolin
UTR	untranslated region
YC	yolk cell
YSL	yolk syncytial layer

Chapter 1

General Introduction

Contents

1 Introduction

During embryonic development, descendants of the single zygote cell diversify into multiple specialized cell types and are arranged into the precise organization of the adult body. The research group in which the present investigations were performed studies the development of a cyprinid teleost fish, the carp (*Cyprinus carpio*). In these studies the following questions are central: How and when are germ layers, in particular the mesoderm, formed? Where do the factors for their induction reside in the embryo and when do they act? When do cells receive information as to the position they will assume on the anteroposterior axis of the embryo?

In addition to examining the morphological processes that take place, we want to address these questions at a molecular level, i.e. by identifying genes that are expressed and by studying how their expression affects the cells involved. In the years preceding the project described in this thesis, very few of the genes involved had been isolated for teleost fish. The aim of the present work was therefore to identify and isolate (novel) genes that are expressed during the above processes, especially during mesoderm formation and during patterning of the anteroposterior axis. The study of where and when a gene is expressed (its expression pattern) is important because it is a step towards elucidation of a gene's function and also because the expression can be used as molecular marker. These aspects are motifs behind the investigations in this thesis.

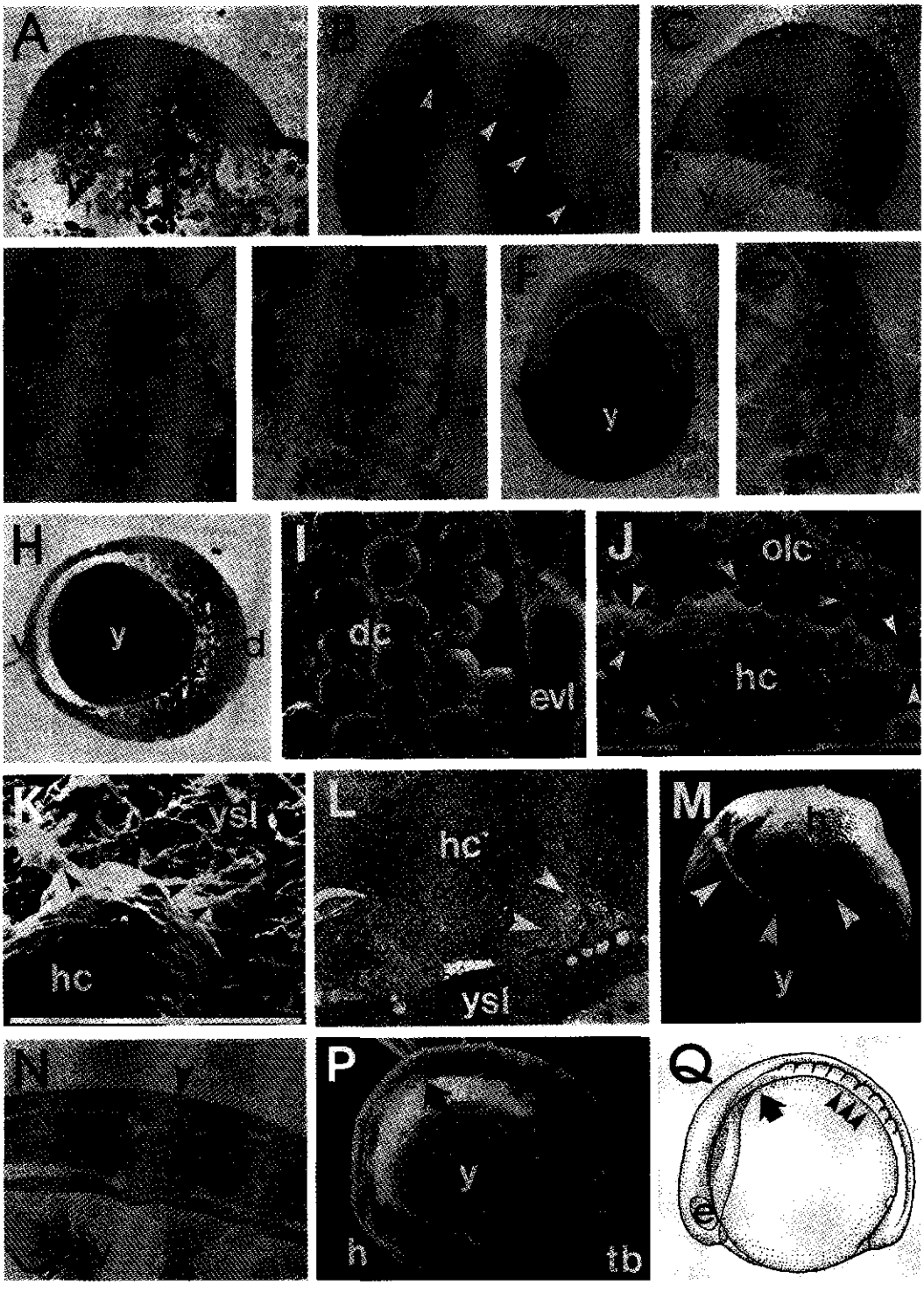
In the following paragraphs it will be first discussed how the carp embryo develops morphologically. Next, attention is given to the formation of mesoderm and endoderm, for the knowledge about these processes has increased almost exponentially from the beginning of this research project (1992) until now (1997). Also the expression and role of the family of homeobox genes are briefly overviewed as they are important regulators of development, and are particularly important for pattern formation on the anteroposterior axis. We have performed a search for these genes because they are important markers of position on this axis. In the final paragraph we explain the experimental approaches that were applied and, lastly, the chapters of this thesis are outlined.

2 Carp early embryonic development

The carp is a cyprinid teleost fish. Some cyprinid teleosts, particularly the goldfish (*Carassius auratus*), the zebrafish (*Danio rerio*), the rosy barb (*Barbus conchonius*) and the carp have been subjects of studies of embryonic development (Stromsten, 1931; Kimmel et al., 1995; Timmermans, 1987; Timmermans and Taverne, 1989; Gevers et al., 1993). In the species examined, the embryo develops in a strikingly similar way. Below, the early development of the carp is described. The intent of

this overview is not to give a full review of all aspects of embryonic development, but to provide sufficient information for the reader to understand the contents of this and other chapters. This description of carp development follows morphological stages of development, hours of development are omitted. For reports of the development of carp at different temperatures, the reader is referred to Neudecker (1976), and Peñáz et al. (1983).

Fig. 1 Early carp development. b = blastomeres, c = cytoplasmic components, d = dorsal, dc = deep cells, e = eye, evl = enveloping layer, h = head, hc = hypoblast cell, n = notochord, olc = outer layer cell, s = somite, tb = tail bud, v = ventral, y = yolk, ysl = yolk syncytial layer. **A** Cytoplasmic streaming at the one cell stage. Cytoplasmic components gather in the animal pole area (movement is represented by arrow on the right) and the yolk platelets move in opposite direction (left arrow), to the vegetal pole. **B** Meroblastic cleavage; blastomeres cleave, but the yolk does not. Note that the blastomeres are still in open contact with the yolk cell (arrowheads). **C** The EVL (arrowheads) forms an epithelial layer that encloses the deep cell mass. **D** The two arrows point at the daughter EVL cell and the daughter deep cell produced after differential cleavage of an EVL cell. Note the mitoses in deep cells (arrowheads). **E** The YSL is the cytoplasmic layer in the animal periphery of the yolk cell (arrowheads). **F** 30-40% epiboly; The blastoderm is spread over the yolk cell. Arrows indicate the direction of movement. **G** By involution of cells from the outer cell layer into the inner cell layer the hypoblast is formed. **H** Convergence; Equatorial section of carp embryo at 100% epiboly. Cells in the epiblast and hypoblast are concentrating on the dorsal side of the embryo. **I** Scanning Electron Microscope (SEM) image of the deep cells and enveloping layer cells at an early epiboly stage. The deep cells are loosely connected (round shape) whereas the EVL cells are tightly packed and form an epithelial layer. **J** SEM image of a hypoblast cell located on the inner surface of the outer cell layer (future epiblast). The cell is in close contact with this layer with its filopodia (arrowheads). **K** A hypoblast cell appears to migrate over the YSL surface, note the filopodial extensions contacting the YSL (arrowheads) (SEM). **L** A Transmission Electron Microscope (TEM) image of part of the hypoblast and the YSL. The asterisk is positioned in one of the YSL nuclei. Some involuted cells have pseudopodia, appearing as light gray areas (arrowheads), and seem to migrate over the YSL. **M** Frontal view of an early segmentation stage embryo folding its head off the yolk. Arrowheads point at head fold. **N** A cross section of the dorsal region of a segmentation stage embryo. The axial mesoderm forms the notochord. On either side of the notochord are the somites. Arrowhead points at the neurectoderm. **P** SEM image of a segmentation stage embryo (side view). The head is to the left and the tail bud to the right. The arrow points at the approximate boundary between head and trunk. In the trunk, somites (see Q) are formed in an anterior to posterior order. **Q** Schematic representation of the segmentation stage embryo in P. Somites are indicated by arrowheads.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2.1 Cleavage

In carp oocytes, cytoplasm and yolk platelets are mingled. Upon contact with water, usually at fertilization, these components are separated. The cytoplasm streams to the future animal pole, while yolk components gather in the vegetal hemisphere of the egg (Fig. 1A). Cleavage starts at approximately 50 minutes after fertilization. The early cleavages last 15 to 20 minutes each. As other teleost eggs (Collazo et al., 1994) carp eggs cleave meroblastically, only affecting the cytoplasm at the animal pole, whereas the large yolk cell remains uncleaved (Fig. 1B). All cells of the blastoderm (blastomeres) stay in open contact with the yolk cell (Fig. 1B) during the first five cleavages which are in animal-vegetal (vertical) directions, perpendicular to each other. Following the 32 cell stage, cleavages also occur in a horizontal plane, thereby partitioning more and more blastomeres off the yolk cell. After approximately six cleavages, some blastomeres (deep cells, DC) are for the first time completely surrounded by other blastomeres and no longer in contact with the outside milieu. Outer cells form the Enveloping Layer (EVL) which by differential cleavage (Fig. 1D) contributes cells to the DC mass and progressively thins into an epithelial-like layer fully covering the DC mass (Fig. 1C and 1I). Conservative cleavages, producing two EVL daughter cells, also occur. The EVL is shed during later development.

Of some cells, especially in the margin of the blastoderm (margin cells), one of the daughter cells remains connected to the yolk cell during subsequent cleavages. After 10 cleavages (approximately 1000 cells), the Yolk Syncytial Layer (YSL, Fig. 1E) arises when these cells fuse to the yolk cell and form a multinucleate cytoplasmic layer at its animal periphery.

There is evidence that maternally derived substances fully control embryonic development until shortly after the tenth cleavage (Stroband et al., 1992). In zebrafish (Kane and Kimmel, 1993), as in *Xenopus* (Newport and Kirschner, 1982), the period in which the embryonic genome starts to be transcribed is called the midblastula transition (MBT). From this period on, embryonic gene products gradually take over the function of maternally supplied proteins and RNA.

2.2 Gastrulation

Gastrulation, consisting of involution and convergence (Van Gestel et al., 1997), is preceded by epiboly. During epiboly the blastoderm is spread over the yolk cell as a thinning sheet of cells (Fig. 1F). This process is completed when the blastoderm margin reaches the vegetal pole. During this period, stages of development are expressed as the percentage by which the yolk cell is covered by blastoderm. Fifty percent epiboly marks the onset of gastrulation. At this stage, deep cells gather in the blastoderm margin, now called the germ ring. The embryonic shield

demarcates the future dorsal side of the embryo as a thickening of the germ ring. Two blastoderm layers arise as cells within the germ ring slip underneath the advancing blastoderm layer (Fig. 1G) and migrate in the direction of the animal pole. At completion of gastrulation, the outer layer is called the epiblast from which the ectoderm and neuroderm are formed. The inner layer, the hypoblast, will give rise to both mesoderm and endoderm and is called the mesendoderm (Thisse et al., 1993). In both layers, cells converge towards the dorsal side (Fig. 1H) and intercalate in the dorsalmost region, causing the cell mass of the embryo to narrow and elongate towards the animal pole. Van Gestel and colleagues (1997) showed that some involuted cells are in contact with the inner surface of the (future) epiblast (Fig. 1J), whereas others contact the YSL (Figs. 1K and 1L). It was suggested that the YSL and the epiblast represent two possible migration pathways for involuted cells.

2.3 Segmentation period

Epiboly is succeeded by the tail bud stage. Most cells are now on the dorsal side (Fig. 1H) of the embryo where the basic body plan of the vertebrate will soon be recognizable. A swelling at the posterior end of the anteroposterior axis marks the tail bud region from which, during the following segmentation period, the tail elongates.

The segmentation period comprises the formation of the somites (Figs. 1P and 1Q), and the number of somites is used to define the developmental stage during this period. Somites form in an anterior to posterior order by segmentation of the paraxial mesoderm on either side of the axial mesoderm (Figs. 1N, 1P and 1Q). The axial mesoderm will form the notochord. Besides skin cells and bone cells, somite tissue differentiates into muscle. In the second half of the segmentation period, muscular contractions appear. In addition to muscle differentiation and formation of organ rudiments, neurulation takes place. From the tail bud stage onwards, the neural plate (Fig. 1M) is visible as a swelling along the anteroposterior axis. The neural plate develops into the neural keel and during its separation from the ectoderm, neural crest cells detach on either side of the keel. The neural keel subsequently takes on a rod-shape and then forms a neurocoele by cavitation. The development of the central nervous system and the differentiation of neurons has been reported in detail for zebrafish by Kimmel and coworkers (reviewed in Kimmel et al., 1995).

In addition to the mesodermal segmentation, the morphological subdivision of the neural tissue becomes visible. In the anterior half of the embryo, the neural tissue forms the brain while in the posterior region it develops as the spinal cord. Swellings in the anterior half of the neural tissue indicate the morphological subdivisions of the brain, the neuromeres. The anteriormost neuromeres correspond to the forebrain

(prosencephalon) and the midbrain (mesencephalon) subdivisions. The posterior half of the brain, the hindbrain (rhombencephalon), is regionalized into rhombomeres.

The segmentation period is followed by the pharyngula period, on the second day of development, and the hatching period in the first hours of the third day. During hatching, the embryo escapes from its chorion, a surrounding protective membrane, and becomes a free swimming larva. The description of these stages is beyond the scope of this thesis. Both stages are well described for zebrafish by Kimmel and colleagues (1995).

3 Origin and induction of mesoderm and endoderm

3.1 Origin of mesoderm and endoderm

One of the intriguing questions of embryonic development is how the three germ layers form from a mass of undifferentiated cells. Our research focuses on the formation of mesoderm and endoderm. In the zebrafish, the endoderm and mesoderm originate from blastomeres in the margin of the blastoderm (Kimmel et al., 1990; Helde et al., 1994; Wilson et al., 1993). The origin and specification of the endoderm was studied by Warga (1996). Endodermal progenitors are located in the three cell tiers closest to the edge by 50% epiboly, whereas mesodermal progenitors are derived from both this area as well as distances further away from the margin (Warga, 1996). The cells that involute early during gastrulation are endodermal as well as mesodermal precursors. The early hypoblast is a single layer, with both precursors intermingled (Warga, 1996). Until midgastrulation (70 to 80% epiboly), hypoblast cells are not irreversibly committed to a hypoblast-derived fate (Ho and Kimmel, 1993). Shortly after midgastrulation, endodermal and mesodermal cells acquire different morphological characteristics. The endodermal cells appear flattened, have filopodia and are in close proximity to the yolk cell, while mesodermal cells are round (Warga, 1996). The cells close to the yolk cell may be similar to those believed to use the YSL as substrate for migration (Fleig, 1990; Van Gestel et al., 1997; see section: 'Gastrulation').

The molecular mechanisms underlying the induction of mesoderm and endoderm have been studied in much greater detail in amphibians (*Xenopus*) than in fish. The mechanisms involved are thought to be conserved between these vertebrate classes (Grunwald, 1996; Stroband et al., 1996a; Mizuno et al., 1996). In *Xenopus*, vegetal blastomeres form endoderm while mesoderm is derived from adjacent (marginal) blastomeres (reviewed in detail by Tiedemann and Tiedemann, 1995; Klein and Melton, 1994; Fukui and Asashima, 1994). Briefly, after fertilization, a rotation of the cortical cytoplasm relative to the deep cytoplasm activates a region in the vegetal blastomeres, the Nieuwkoop center. The Nieuwkoop center emits dorsalizing signals to overlying marginal blastomeres. Ventral vegetal

and lateral vegetal blastomeres provide ventralizing signals to overlying marginal blastomeres that induce these to ventral mesoderm fates. Dorsal marginal blastomeres are induced to dorsal mesoderm and, in addition, become the Spemann organizer that emits signals that organize the embryonic axis (see Lemaire and Kodjabachian, 1996). Dorsalizing signals from the organizer refine the dorsoventral pattern of mesoderm at lateral positions.

3.2 Origin of inductive signals

Mesoderm induction and patterning are events closely linked to dorsoventral axis formation (Asashima, 1994). In the living zebrafish embryo, the dorsal side is not apparent before 30% epiboly (Schmitz and Campos-Ortega, 1994). In contrast, the position of the future dorsal side in *Xenopus* is fixed upon fertilization, opposite the sperm entry point (SEP) (Gerhart et al., 1989). The teleost SEP is located near the animal pole (Wacker et al., 1994; Linhart et al., 1995) and is therefore not predictive for the position of the dorsal side. Moreover attempts to, for example, correlate the future dorsoventral axis to directions of the first cleavage planes appeared vain (Kimmel and Law, 1985; Strehlow and Gilbert, 1993; Wilson et al., 1993; Helde et al., 1994; Abdelilah et al., 1994; Wacker et al., 1994).

It has been postulated that, like in *Xenopus*, mesoderm induction and patterning in teleosts requires signaling from early cleavage stages onwards by maternally derived components, which, in fish, are thought to be present in the yolk cell cytoplasm and from there transferred to the marginal blastomeres (reviews by Grunwald, 1996; Driever, 1995; Stroband et al., 1996a). Recent studies provide more and more support for the correctness of this hypothesis. Mizuno et al. (1996) demonstrated that zebrafish yolk cells that were transplanted onto the animal pole region of host embryos induce ectopic expression of a dorsal and a pan-mesodermal gene marker, further underlining that mesoderm-inducing signals are derived from the yolk cell and passed on to the blastomeres. The cytoplasmic components required for formation of dorsal mesoderm and other dorsal structures were recently shown to reside in the vegetal hemisphere of the yolk cell during the first cleavages (Mizuno et al., 1997; Jesuthasan and Strähle, 1996), thus resembling *Xenopus*. The initial dorsoventral asymmetry in zebrafish appears to depend on a region of specific microtubule structures near the vegetal pole (Jesuthasan and Strähle, 1996). Polystyrene beads were used to mimic the movement behavior of the yolk cell cytoplasmic granules (Jesuthasan and Strähle, 1996), which are supposed to contain mesoderm inducing signals. By microtubules, material in the yolk cortex is transported from the vegetal hemisphere in the direction of the animal pole. Inductive signals reach the blastomeres after the 32 cell stage (Mizuno et al., 1997). At the 1000 cell

stage, the blastoderm is capable of mesoderm formation in absence of the yolk cell (Bozhkova et al., 1994), but correct pattern formation requires signaling from the yolk cell that occurs beyond this stage (Bozhkova et al., 1994; Cooper and D'Amico, 1996). Evidence exists for communication between yolk cell and blastoderm cells in later stages of development (Cooper and D'Amico, 1996; Gevers and Timmermans, 1991).

3.3 Molecular nature of inductive signals[1]

Potential candidates for signaling leading to axis formation and dorsal mesoderm induction are members of the TGFß and wnt families (Kessler and Melton, 1994; Moon et al., 1997; Slack, 1994). In *Xenopus*, the Nieuwkoop center signaling depends on an intracellular signal transduction pathway, activated by a wnt factor (Wnt8; Fagotto et al., 1997; Brannon and Kimelman, 1996; Schneider et al., 1996). It seems that an equivalent signaling pathway plays a role in the zebrafish (Kelly et al., 1995a, 1995b; Schneider et al., 1996). In *Xenopus* this signaling targets *Siamois*, a homeobox gene in the dorsal inducing pathway of the Nieuwkoop center (Lemaire et al., 1995; Carnac et al., 1996; Brannon and Kimelman, 1996). *Siamois* expression is activated after maternal ß-catenin protein translocates into the nuclei of the Nieuwkoop center in the frog (Larabell et al., 1997; Schneider et al., 1996; Fagotto et al., 1997; Moon et al., 1997 and references therein). In zebrafish such nuclear ß-catenin translocation, likely indicative of a fish primary dorsalizing center alike the *Xenopus* Nieuwkoop center, has been observed in a region of the YSL (Schneider et al., 1996).

TGFß family members involved in dorsal mesoderm induction are for example activin (Gotoh and Nishida, 1996) and Vg1 (DVR-1 in zebrafish) (Dohrmann et al., 1996; Helde and Grunwald, 1993). Activin is a potent inducer and essential for the induction of mesoderm in both fish and *Xenopus* (Wittbrodt and Rosa, 1994; Dyson and Gurdon, 1997).

FGF family members are generally considered inducers of ventro(lateral) mesoderm in *Xenopus* (Fukui and Asashima, 1994; Slack, 1994; Kimelman, 1993), but are nevertheless also capable of inducing dorsal mesoderm (Gotoh and Nishida, 1996; Kimelman, 1993). FGF and activin interact, probably through dependence of activin on functional FGF signaling (Gotoh and Nishida, 1996). The ventral mesodermal fate was in the past considered a 'default' state, being formed in the absence of dorsalizing signals. Recently the existence of ventralizing signals and their requirement in the formation of ventral structures became clear (Gotoh and Nishida, 1996; Mullins et al., 1996; Hammerschmidt et al., 1996). BMPs (Bone Morphogenetic proteins), possibly BMP4 or BMP4/BMP7

[1]Protein names are written upright whereas the names of genes and mRNAs are in *italics*

heterodimers, are candidates for ventralizing factors (Hogan, 1996; Suzuki et al., 1997; Nikaido et al., 1997).

3.4 Immediate early response genes expressed in mesoderm

Vegetal signals activate the expression of a number of immediate early response genes (i.e. genes expressed in the presence of an inhibitor of protein synthesis) in the mesoderm. Activin and FGF for example induce *Xbra/no tail* (*ntl*) (Gotoh and Nishida, 1996). In zebrafish, ntl protein is expressed in nuclei of the germ ring and the notochord (Schulte-Merker et al., 1992). This protein functions as activator of transcription of mesoderm-specific genes (Conlon et al., 1996). *Goosecoid* (*gsc*) is activated by activin (Joore et al., 1996) and is expressed in the first involuting cells that will form the axial hypoblast anterior to the notochord (Stachel et al., 1993; Thisse et al., 1994). Posterior to the *goosecoid* expressing cells, the axial mesoderm expresses *floating head*, a gene necessary for development of the notochord (Talbot et al., 1995; Melby et al., 1996). *Lim1* is initially expressed in the entire margin of the zebrafish blastoderm and becomes restricted to the axial mesoderm during gastrulation (Toyama et al., 1995). Two novel genes important for mesoderm formation in *Xenopus* were described by Ryan et al. (1996) and Stennard et al. (1996). *Eomesodermin* (*Eomes*) and *Antipodean* (*Apod*), both members of the T-box family of transcription factors, are the earliest known genes expressed in the frog mesoderm, in response to vegetal signaling. The genes can regulate each others transcription as well as that of other mesodermal genes, for example *Xbra*. *Eomes* is supposed to have a key role in initiating mesoderm differentiation and in determining mesodermal cell fate (Ryan et al., 1996).

3.5 Endoderm

Though not much is known about the factors that induce endoderm in teleosts and in *Xenopus*, molecular studies suggest that endoderm formation may occur by the same signaling mechanisms that mediate mesoderm induction and patterning. For example peptide growth factors of the TGFß (activin, Vg1) and FGF families induce frog animal cap explants (which normally do not form endoderm) to express endoderm-specific markers such as 4G6 (Jones et al., 1993), Xlhbox8 en IFABP (Henry et al., 1996). In addition, *chordin* and *noggin*, factors secreted by the organizer, induce the expression of *endodermin*, an pan-endodermal marker, probably by opposing BMP signaling (Sasai et al., 1996). In zebrafish, endodermal and mesodermal precursor cells are difficult to distinguish (see section 'Origin of mesoderm and endoderm'). According to Warga (1996) endoderm and mesoderm become morphologically distinct shortly after midgastrulation. Only few molecular markers recognize early zebrafish endoderm cells; FKH1 protein after 60% epiboly (Warga, 1996),

and *axial* after midgastrulation (Schier et al., 1997).

4 Homeobox genes

4.1 Clustered homeobox genes and specification of the anteroposterior axis

For spatially correct differentiation of mesodermal, endodermal and ectodermal derivatives it is necessary that cells receive information about their position on the anteroposterior axis. A group of genes that specify regional identities along this axis are the *Hox* genes (Keynes en Krumlauf, 1994; Maconochie et al., 1996, and references therein). Because of their overlapping expression on the anteroposterior axis (discussed below), the transcripts of these genes are appropriate markers of anteroposterior position.

The *Hox* genes belong to the superfamily of homeobox genes, which is characterized by the presence of a homeobox. The homeobox encodes the homeodomain, a conserved motif of 60 amino acids which mediates sequence specific DNA binding (Gehring et al., 1994). Homeodomain proteins regulate the transcription of target genes (Gehring, 1994). This motif was first identified in *Drosophila* genes which regulate the identity of the body segments during development (Lewis, 1978). In this species, a number of homeobox genes are organized in two complexes on the same chromosome, *Bithorax* and *Antennapedia,* collectively known as the *HOM* complex (McGinnis and Krumlauf, 1992). Since their first discovery, homologs of the *Drosophila HOM* genes have been identified in most species examined, ranging from fungi, plants to animals (Ruddle et al., 1994). In vertebrates, including teleosts, the homologs of the *HOM* genes are called *Hox* genes and are present as four clusters on four different chromosomes (reviewed in Krumlauf et al., 1993; Misof et al., 1996; van der Hoeven et al., 1996). It appears that the *Hox/HOM* genes have arisen by duplication and divergence of a single ancestral cluster (Ruddle et al., 1994), which in vertebrates yielded four *Hox* clusters (*Hoxa*, *Hoxb*, *Hoxc* and *Hoxd*), each containing 13 genes at most (Krumlauf, 1994; Misof et al., 1996). Genes at similar positions within each cluster share most homology and are considered paralogous groups.

expression and functional domains

The spatial and temporal expression of *Hox* genes is well conserved across species, and shows a colinear relationship to their organization within a chromosomal cluster (Lewis, 1978; Krumlauf et al., 1993). Though *Hox* genes are expressed in the mesoderm (and probably endoderm) as well as in the ectoderm, their expression in neural tissue in general receives most attention. The sequence of expression of *Hox* genes in overlapping domains along the anteroposterior axis of the hindbrain and

spinal cord largely follows their order on the chromosome. Generally, genes at 3' position in the cluster are expressed early, and their anterior limits of expression are in the hindbrain. Towards the 5' end of the cluster, genes are expressed at progressively later times and their anterior expression limits reach less far anterior than those of their 3' neighbors. *Hoxb-1* and *Hoxb-2* are exceptions to the colinearity rule since the anterior limit of *Hoxb-2* expression is anterior to that of *Hoxb-1* (Keynes and Krumlauf, 1994).

Mutational studies suggest that the functional domains of *Hox* genes usually correspond to their anterior-most domains of expression (Maconochie et al., 1996). In principle, the anterior border of expression of paralogous groups 1-4 are in the hindbrain and those of groups 5-13 in the spinal cord (Krumlauf et al., 1993). The anterior limits of expression of the paralogous groups 1-4 genes coincide with the segmental organization of the hindbrain, the rhombomeres (r) (Keynes and Krumlauf, 1994). Members 1-4 of for example the mouse *Hoxb* cluster have their anteriormost expression in r4, r3, r5, r7 respectively (Krumlauf et al., 1993). It is most likely that the *Hox* genes are not the segmentation genes themselves, but instead specify the rhombomere phenotype (Keynes and Krumlauf, 1994).

How the domains of *Hox* gene expression are established is a question for which no clear answers yet exist. A few possible regulators have been identified, for example *Krox-20* regulates *Hoxb-2* (Guthrie, 1996; Keynes and Krumlauf, 1994). There is evidence suggesting that retinoic acid (RA, a vitamin A derivative) is an endogenous morphogen that generates the boundaries of the *Hox* expression (for discussion see reviews by Durston et al., 1996; Marshall et al., 1996; Conlon, 1995). Also, paralogous, non-paralogous *Hox* genes and other transcription factors interact, forming a complex regulatory network (for discussion see Guthrie, 1996; Krumlauf, 1994; Duboule and Morata, 1994).

4.2 Non-clustered homeobox genes

By divergence many homeobox-containing genes evolved that are not located in the chromosomal clusters (Ruddle et al., 1994; listed in Stein et al., 1996). These non-clustered homeobox genes[2] are grouped in different classes, for example *prd* (*paired*), *POU* and *caudal*-type (Bürglin, 1994). The non-clustered genes have important functions as transcription factors in many aspects of development. For example *Siamois*, *floating head* and *goosecoid*, involved in embryonic axis formation (see sections: 'Molecular nature of inductive signals' and 'Immediate early response genes expressed in mesoderm'), are homeobox genes like many other organizer-

[2] '*Hox* genes' denotes the vertebrate clustered homeobox genes. The description 'homeobox genes' refers to both clustered and non-clustered homeobox genes.

expressed genes (Lemaire and Kodjabachian, 1996).

In the anterior brain and head, where *Hox* genes are not expressed, the combinatorial expression of non-clustered homeobox genes such as *otx* and *dlx* may specify position (Bürglin, 1994; Finkelstein and Boncinelli, 1994; Akimenko et al., 1994; Li et al., 1994; Mori et al., 1994).

Not only the *Hox* genes provide positional information, non-clustered homeobox genes also appear to play a role in anteroposterior patterning (Pownall et al., 1996). *Carp-cdx1/zebrafish-cad1* (*caudal*-type genes) and *eve1* are expressed in the posterior region of the gastrulating fish embryo (Joly et al., 1992; Joly et al., 1993; this thesis). Overexpression of a *caudal*-type gene in *Xenopus* (*Xcad3*) leads to anterior truncations, as does FGF overexpression (Pownall et al., 1996). It has been demonstrated that FGF and *Xcad3* regulate the normal expression of posterior *Hox* genes (Pownall et al., 1996). Like *Xcad3* in *Xenopus*, overexpression of *eve1* in zebrafish affects anterior structures (Barro et al., 1995).

5 Scope of the thesis

For the molecular identification of genes, in particular *novel* genes, that relate to mesendoderm formation we chose a subtractive hybridization strategy. This strategy enabled us to isolate genes that are differentially expressed between the oocyte stage and the early segmentation stage, i.e. expressed in one stage but not in the other. Such differentially expressed genes are of interest because they may be involved in stage specific processes like determination, differentiation and morphogenesis. As one of the stages to be subtracted, the oocyte stage was chosen. Maternally derived proteins and mRNAs in the oocyte are responsible for embryonic development up until at least MBT (when the embryonic genome is activated) and they are involved in the early events of mesendoderm induction. In the second stage (early segmentation), after completion of gastrulation, the basic vertebrate body plan has been established and the differentiation of the germ layers is in progress.

Subtractive hybridization (Sargent, 1987; Wang and Brown, 1991) of the cDNA fragments of one stage with those of the other stage removes the common abundantly expressed sequences representing for example housekeeping genes. It is done by hybridizing the cDNA fragments of the developmental stage of interest with biotin-labeled cDNA fragments from the second stage. After hybridization, the biotin-containing cDNA hybrids are removed (subtraction), leaving behind the cDNA fragments from which the common fragments with the second developmental stage have been largely eliminated. For the production of enriched probes of the oocyte stage, cDNA fragments of this stage were subtracted with the cDNA fragments of the segmentation stage. Vice versa, the early segmentation stage was subtracted with the oocyte stage. The enriched probes were

A

RRRKRTAYTRYQLLELEKEFHFNRYLTRRRRIELAHSLNLTERQVKIWFQNRRMKWKKEN

forward primer reverse primer

B

cdx1	KEKYRVVYTDHQRLELEKEFHFNRYITIRRKSELAVNLGLSERQVKIWFQNRRAKERKLI
ovx1	SRRRRTAFTSEQLLELEKEFHCKKYLSLTERSQIAHALKLSEVQVKIWFQNRRAKWKRIK
Hoxb-1	QNTIRTNFTTKQLTELEKEFHFSKYLTRARRVEIAATLELNETQVKIWFQNRRMKQKKRE
Hoxb-3	SKRARTAYTSAQLVELEKEFHFNRYLCRPRRVEMANLLNLSERQIKIWFQNRRMKYKKDQ

Fig. 2 A Consensus sequence of the homeodomains of proteins encoded by homeobox genes (derived from 346 homeodomain sequences; Bürglin, 1994). PCR primers were designed against the most conserved regions of the homeodomain; 'LELEKEFH' and 'KIWFQNRR' (underlined), and as a result bind to the homeobox sequences of different homeobox genes. By PCR a mixture of homeobox fragments is produced. This mixture was used to isolate the corresponding homeobox genes from the carp early segmentation stage cDNA library. **B** Homeodomains of cdx1 (Chapter 2), ovx1 (Stroband et al., 1996b), Hoxb-1 (Chapter 3) and Hoxb-3 (In Der Rieden et al., 1996).

used for the identification of differentially expressed genes in the cDNA libraries of the two stages (differential plaque hybridization, Almendral et al., 1988).

The subtractive hybridization approach is particularly fitted to isolate novel, differentially expressed genes, but is not a straightforward method for the isolation of members of specific gene families. Because we wished to isolate genes involved in anteroposterior patterning, we specifically searched for homeobox-containing genes. The isolation method that we followed was founded on the conservation of the homeodomain sequence. In the consensus sequence of the homeodomain (Fig. 2) described by Bürglin (1994) and derived from 346 homeodomain proteins, the most conserved regions were identified and used to design PCR primers. In principle, these primers bind to all genes that possess the conserved motifs of the consensus sequence, thus to both clustered and non-clustered homeobox genes. By PCR we obtained a mixture containing homeodomain fragments of several different homeobox genes and this mixture was used as a probe to screen the carp segmentation stage cDNA library for homeobox genes.

outline

Chapter 2 describes the screening for homeobox-containing genes and describes the sequence and mRNA expression pattern of one of the isolated genes, *cdx1*, a non-clustered homeobox gene. Chapter 3 deals with the description of another gene identified by this method, *Hoxb-1*, belonging to the clustered genes and expressed in a restricted domain of the anteroposterior axis. *Hoxb-1* expression marks a region of the hindbrain, rhombomere 4. The results of the subtractive hybridization strategy are presented in Chapter 4. In Chapter 5, the distribution of the transcripts of the *cth1* gene, isolated by subtractive hybridization, is examined. The hypothesis is presented that this novel gene could be involved in preventing determination during early development. Chapter 6 provides a short review of the literature dealing with the class of C3H genes, to which the *cth1* gene belongs.

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Chapter 2

Expression of carp-cdx1, a caudal homolog, in embryos of the carp, Cyprinus carpio

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Abstract

A carp *caudal* cDNA of 1.3 kb was cloned after screening an early segmentation stage cDNA library with a probe produced by PCR using conserved homeobox sequences as primers and genomic DNA as template. The homeobox gene was called *carp-cdx1*. The gene appears highly similar to other vertebrate *caudal* homologs, especially the zebrafish gene *cdx [Zf-cad]*. The possible relationship to homeobox genes within the *Hox* gene complexes is discussed. A weak expression of the gene, detected by in situ hybridization, was found shortly before gastrulation (at 25% epiboly) in cells likely to have a posterior fate. During gastrulation expression became stronger. At the early segmentation stage, cells of the neural keel in the area of the prospective spinal cord expressed the gene. During the progression of segmentation, expression retracted in caudal direction. The tail bud expressed the gene throughout, but the somites lost expression shortly after their formation. Only the most lateral mesoderm cells maintained expression in the trunk area. *Carp-cdx1* was also expressed in the endoderm. At 24 hours after fertilization the gene was only expressed in the tail bud. At 48 hours, no expression could be detected. The expression pattern suggests a function for *carp-cdx1* in gastrulation and patterning along the anteroposterior axis of the embryo.

Introduction

The zebrafish is widely used in developmental studies as a model for vertebrate development, but we have chosen the carp for our study of early fish development. Common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*) are both cyprinid teleosts. Comparison of morphogenesis and gene expression patterns in zebrafish and carp may give information on the degree of generality of results found in zebrafish and may make possible discrimination between essential and less essential features.

Among genes regulating early embryonic development, homeobox genes are extremely interesting, since they contain a very conserved sequence, the homeobox, coding for a DNA-binding part of the protein, which functions as a transcription factor (Scott et al., 1989). These genes appear to be key elements in the development of the bodyplan during ontogeny and phylogeny (Graham et al., 1989; Holland, 1992; McGinnis and Krumlauf, 1992; Pendleton et al., 1993; McGinnis and Kuziora, 1994).

Homeobox genes were first detected in *Drosophila*, and a number of these genes appeared clustered on one chromosome forming the *Antennapedia* and the *Bithorax* complexes (Kaufman, 1983), together termed the Homeotic complex (*HOM* complex; McGinnis and Krumlauf, 1992). In many other insects, the two clusters are not separated and form one *HOM* cluster. Within

these complexes, the order of the genes along the chromosome is represented by their expression domains along the anteroposterior (a-p) axis (Kaufman et al., 1990). In vertebrates, usually four *Hox* complexes are found and a similar relationship between the localization within a gene complex and the pattern of expression exists (Graham et al., 1989; Duboule and Dollé, 1989; McGinnis and Krumlauf, 1992; Holland, 1992). It is supposed that the vertebrate *Hox* complexes have arisen from duplication of an ancestral homeobox gene complex (Hunt and Krumlauf, 1992). This is reflected by the presence of less than four complexes in more primitive chordates (Pendleton et al., 1993). Structural comparison of homeobox genes of vertebrates and arthropods suggests that their common evolutionary ancestor had one complex of a small number of homeobox genes (McGinnis and Krumlauf, 1992). Duplication of homeobox genes also may have led to the presence of a number of genes not or no longer linked to the *HOM/Hox* complexes. These homeobox genes may also be involved in axial patterning of the embryo.

In an attempt to find carp homeobox genes with expression during early development, we isolated gene clones from an early somite stage cDNA library using a polymerase chain reaction (PCR) probe produced with the most conserved sequences of *Antennapedia* as primers. Among the cloned genes, one appears highly similar to the zebrafish *caudal* gene (*Zf-cad1*; Joly et al., 1992). Homologs of *caudal*, first described for *Drosophila* (Mlodzik et al., 1985), were also found in *C. elegans* (Bürglin and Ruvkun, 1993), *Bombyx* (Xu et al., 1994), *Amphioxus* (Holland et al., 1994), *Xenopus* (Blumberg et al., 1991; Northrop and Kimelman, 1994), chicken (Frumkin et al., 1991), mouse (Duprey et al., 1988; Meyer and Gruss, 1993; Gamer and Wright, 1993) and rat (Freund et al., 1992). These genes do not belong to the *HOM* or the *Hox* complexes but reside elsewhere in the genome. In *Drosophila*, a posterior to anterior gradient of *caudal* gene product exists during early development and, in all vertebrate species studied so far, differential expression of *caudal* along the a-p axis is found: the gene is merely expressed in the caudal part of the embryo. This paper focuses on the sequence analysis of cDNA of the carp *caudal*-homolog (named *carp-cdx1*), comparison of this sequence and the deduced protein sequence to those of other vertebrate species (especially the zebrafish), and on the pattern of *carp-cdx1* expression during development. The possible relationship between *caudal* homologs and genes within the *HOM* and the *Hox* complexes is discussed.

Results

Sequence analysis of carp-cdx1

Screening a carp early somite stage cDNA library with a general genomic *Antennapedia* derived homeobox probe revealed 26 positive clones. So far,

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1                                                                   GATAAATAGTATTGAGTCAGGCCTTG
                                                  *         *         *
27    GTTTTGAGTAGGATATTTGGCTTGATGTATGGCTGGCATGTTGCCTTAAAAAAAGTTTTCTTAGGGGTTTCTTTCGAATC

106   ATG TAC GTT GGA TAC CTT TTG GAT AAA GAG GGA AGC ATG TAT CAC CAA GGA TCC GTG CGA
1      M   Y   V   G   Y   L   L   D   K   E   G   S   M   Y   H   Q   G   S   V   R

166   AGA TCT GGA ATC AGT CTT CCA CCA CAG AAT TTT GTC TCC ACA CCA CAG TAT TCA GAT TTT
21     R   S   G   I   S   L   P   P   Q   N   F   V   S   T   P   Q   Y   S   D   F

226   ACA GGA TAC CAT CAT GTG CCC AAC ATG GAC ACA CAC GCG CAG TCA GCG GGA GCA TGG GGC
41     T   G   Y   H   H   V   P   N   M   D   T   H   A   Q   S   A   G   A   W   G

286   CCT CCT TAC GGC GCA CCG AGA GAG GAC TGG GGC GCC TAC AGC CTG GGA CCT CCA AAC ACT
61     P   P   Y   G   A   P   R   E   D   W   G   A   Y   S   L   G   P   P   N   T

346   ATT TCT GCA CCT ATG AGC AGT TCA TCC CCG GGA CAA GTT TCC TAC TGC TCT TCA GAT TAT
81     I   S   A   P   M   S   S   S   S   P   G   Q   V   S   Y   C   S   S   D   Y

406   AAT GCC ATG CAC GGG CCG GGA TCA GCG GTG CTG CCT CCA CCA CCT GAA AAC ATT TCT GTG
101    N   A   M   H   G   P   G   S   A   V   L   P   P   P   P   E   N   I   S   V

466   GGC CAG CTT TCA CCT GAG AGA GAA AGA CGC AAT TCC TAT CAG TGG ATG AGC AAA ACG GTG
121    G   Q   L   S   P   E   R   E   R   R   N   S   Y   Q   W   M   S   K   T   V

526   CAG TCC TCA TCA ACC GGC AAA ACG AGA ACG AAG GAG AAA TAT CGA GTG GTG TAC ACA GAC
141    Q   S   S   S   T   G   K   T   R   T   K - E - K - Y - R - V - V - Y - T - D

586   CAT CAG AGG TTG GAG CTT GAG AAA GAA TTT CAT TTT AAT CGC TAC ATC ACA ATC AGA AGA
161    H - Q - R - L - E - L - E - K - E - F - H - F - N - R - Y - I - T - I - R - R

646   AAA TCC GAA TTG GCA GTA AAC CTC GGG CTT TCA GAA AGA CAG GTA AAG ATT TGG TTT CAG
181    K - S - E - L - A - V - N - L - G - L - S - E - R - Q - V - K - I - W - F - Q

706   AAC CGC AGA GCT AAG GAA AGG AAA TTA ATC AAA AAG AAG CTG GGT GTG TCT GAC GGC AGC
201    N - R - R - A - K - E - R - K - L - I   K   K   K   L   G   V   S   D   G   S

766   GGA GGA TCA GTT CAC AGC GAT CCC GGT TCT GTC AGT CCC CTA CCG GTA CCG GGG GTC ACT
221    G   G   S   V   H   S   D   P   G   S   V   S   P   L   P   V   P   G   V   T

826   GAG TCC GTC AGA CAT ACA CGG TTC TCT GTA CCC ACC CCC AGG AAT GAA CGC CTT ACC ATC
241    E   S   V   R   H   T   R   F   S   V   P   T   P   R   N   E   R   L   T   I

886   TAT GAG GAA CAT ACA GCA AGT TAC TGT CAC TCA GTG AAC TCG AAC TTG AAA GAT CCA ACT
261    Y   E   E   H   T   A   S   Y   C   H   S   V   N   S   N   L   K   D   P   T

946   TTA ACT GAG CAC TCC AAC ACG CTG AAA GGA CAG AAC CGA AGC GGA CCG CTC GCG TGG ACA
281    L   T   E   H   S   N   T   L   K   G   Q   N   R   S   G   P   L   A   W   T

1006  AAA CGG ATT ACA AAC TAT TAC ACG ACA GGC ATC AAG GCG CGC GTT GTG GAT TAC CAC AGA
301    K   R   I   T   N   Y   Y   T   T   G   I   K   A   R   V   L   D   Y   H   R

1066  AGA ATC ACG TCT TAAATGCAACGAAATTCTGGAGGCTCTTCAAATATTGTTTTAATAGAATAGTTTTTCAAATTA
321    R   I   T   S  END

1141  AGAAAAGAAAAAAAAAAACGTCTGGTTCGTCTGATCCAGAGACTAAATAATTTTAATGGTTTGTTTGTGTTTGCTCAATT

1220  AAGATATAAATATCATTTTCTGTATGTGGTGAGTGTATCTAAATGTTTGTATATAAAATGAAACTACAAGTAAAAAAAA

1299  AA
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Fig. 1 cDNA sequence and deduced amino acid sequence of the carp homeobox gene *carp-cdx 1*. Upstream initiation and termination codons are printed in bold and marked with an asterisk; the homeobox is printed in bold; the amino acids of the homeodomain are connected by a hyphen; the five conserved regions specific for *caudal* class genes are underlined.

four clones have been (partially) sequenced and all appear to represent homeobox genes. One of those, a 1.3 kb clone, (Fig. 1) contains 105 nucleotides of the 5′ untranslated region, an open reading frame encoding a putative protein of 324 amino acids (calculated relative molecular mass 39 x 10^3) and an untranslated 3′ end of 214 nucleotides and a poly-A tail. At positions of 14 and 17 triplets upstream from the supposed initiation codon, other in frame ATG sequences are present. However, their contribution to initiation of translation would be very weak compared to that of the first Met (M) codon, which is part of an optimal initiation sequence (an A at position minus 3 from the M codon; Kozak, 1986). Furthermore, an in frame stop codon is present 11 triplets upstream from the initiation M codon. At position 51, an M codon is present in an optimal initiation sequence. However, a stop codon follows at position 87. Between positions 556 and 735 a homeobox sequence is present.

Sequence analysis reveals that the gene is highly similar to *caudal*-like genes described for other vertebrates, especially the zebrafish (Table 1). Therefore, it was named *carp-cdx1* in accordance with suggestions about nomenclature of homeobox genes by Scott (1992). All over basepair identity for the coding region is highest (88%) for zebrafish *Zf-cad1*, and the same holds for all over deduced amino acid identity (93%). For the homeobox these figures are even higher (97% and 100% respectively).

The deduced carp-cdx1 protein furthermore contains 5 highly conserved areas apart from the homeobox (Fig. 1). These amino acid sequences appear specific for all vertebrate caudal proteins known (Gamer and Wright, 1993). The 1.4 kb *caudal* cDNA of *Drosophila* (Mlodzik and Gehring, 1987) codes

Table 1 Percentage of identity between homeoboxes of *carp-cdx1* and other *caudal* homologs

	Nucleotides	Amino acids
Dros. caudal	63	80
Bombyx-cad	77	89
Zf-cad1	97	100
Xcad1	-	87
Xcad2	-	85
Xcad3	72	80
CHox-cad	72	85
Cdx-1	73	87
Cdx-2	72	85
Cdx-4	79	95
Rat Cdx	71	87

Sources: as Fig. 2

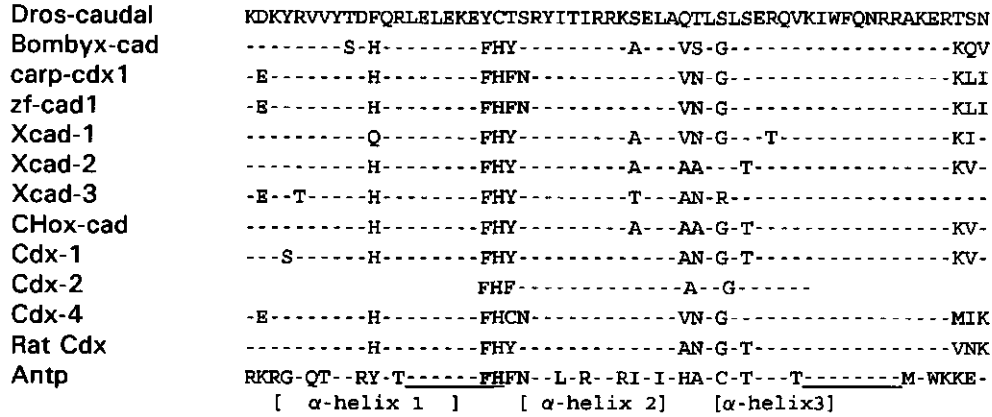
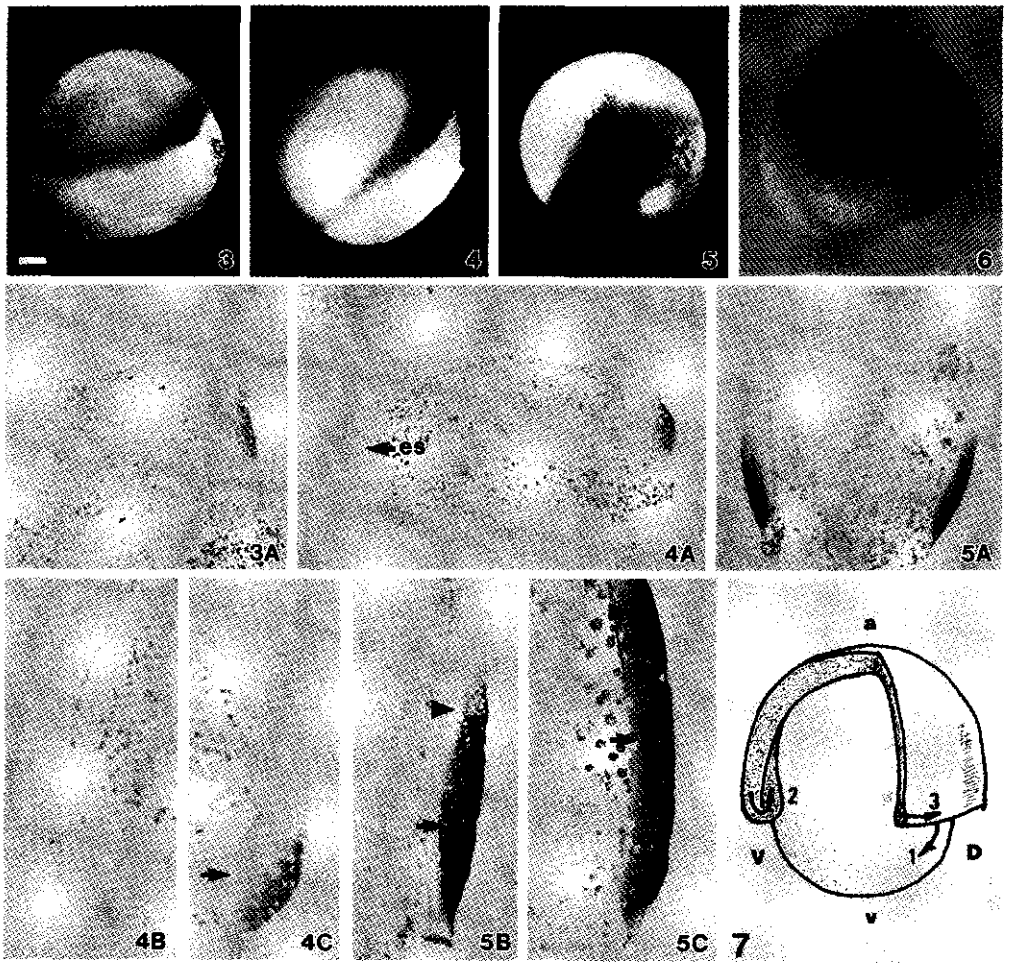
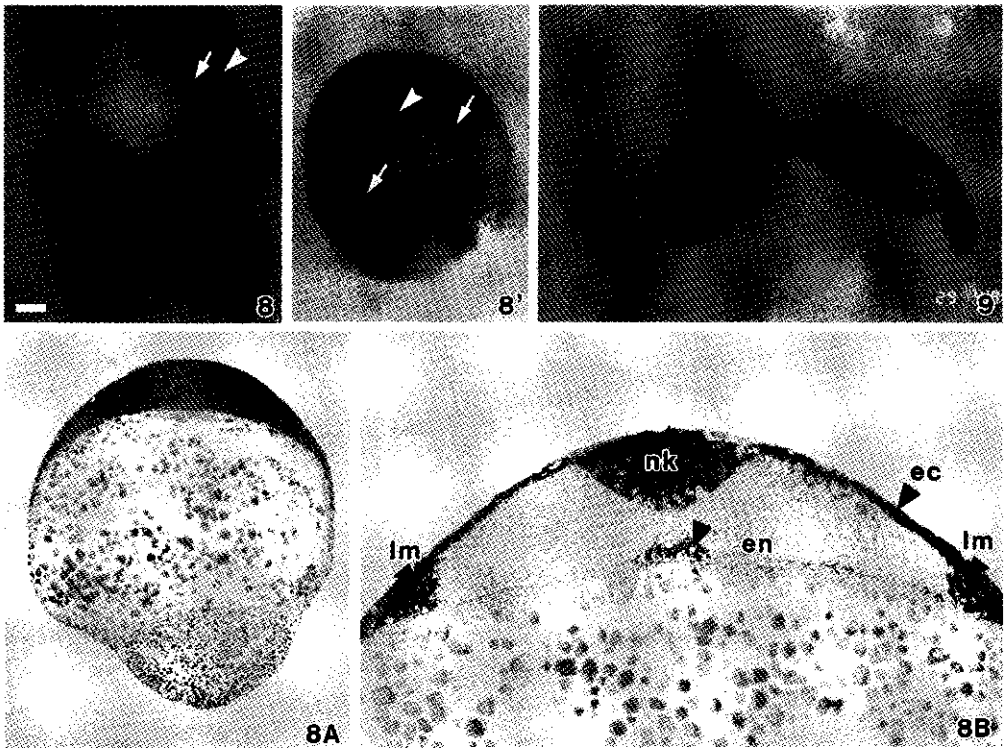


Fig. 2 Comparison of homeodomain sequences of different *caudal* homologs and *Drosophila Antennapedia*. α-helical sequences are indicated by brackets; the regions of primers, used for PCR, are underlined. Sources: *Drosophila* caudal: Mlodzik et al. (1985); Bombyx-cad: Xu et al. (1994); zebrafish zf-cad1: Joly et al. (1992); *Xenopus* Xcad-1/2: Blumberg et al. (1991); Xcad-3: Northrop and Kimelman (1994); Chick CHox-cad: Frumkin et al. (1991); Mouse Cdx-1: Duprey et al. (1988); Cdx-2: James and Kazenwadel (1991); Cdx-4: Gamer and Wright (1993); *Rat* Cdx: Freund et al. (1992); *Drosophila* Antennapedia: Garber et al. (1983).

Figs. 3-7: Epiboly and gastrulation. Bar = 140μm (Figs. 3, 4, 5); 130 μm (Fig. 6); 110 μm (Figs. 3A, 4A, 5A); 40 μm (Figs. 4B, 4C; Figs. 5B, 5C). **3** Whole mount in situ hybridization of 7 hours old embryo at 25% epiboly. *Carp-cdx1* expression product (brown) is unevenly distributed in the vegetal area of the blastoderm. **3A** Section of embryo in Fig. 3, showing expression on the right, but not the left part of the blastoderm. **4** Whole mount in situ hybridization at 50% epiboly. Note the uneven distribution of *carp-cdx1* expression, being strongest ventrally, opposite the embryonic shield (see Figs. 4A, 4B and 4C). **4A** Section of embryo in Fig. 4. The embryonic shield (es) contains less label than the opposite (ventral) area of the blastoderm. **4B** Section of embryo in Fig. 4. The area including the embryonic shield does not show gene expression. **4C** Same section as Fig. 4B. The ventral area (opposite embryonic shield) is strongly labeled. Arrow: non-labeled involuted cells. **5** Whole mount in situ hybridization at 95% epiboly. Cells around the yolk plug are labeled. **5A** Section of embryo at 80% epiboly, showing expression of *carp-cdx1* in a broad zone of marginal blastomeres. **5B** Same section as in Fig. 5A. Label is present in epiblast and, to a lesser extent, in recently involuted hypoblast cells



(Figs. 3-7 continued)
(arrow). Early involuted cells are negative (arrowhead). **5C** Section of embryo of Fig. 5 (95% epiboly). Label is present in epiblast and, to a lesser extent, in involuted hypoblast cells (arrow). Earlier involuted cells are negative (arrowhead). **6** Whole mount in situ hybridization of embryo after completion of epiboly, showing gene expression only in the caudal part of the embryo. **7** Schematic drawing of carp embryo showing the three principle directions of cell movements: epiboly (1), involution (2) and convergence (3). a = animal pole; v = vegetal pole; V = ventral; D = dorsal.



Figs. 8-9: segmentation stage embryos. Bar = 130 μm (Figs. 8, 9), 60μm (Fig. 8A), 35 μm (Fig. 8B). **8** Whole mount in situ hybridization of embryo at 14 hours after fertilization (a.f.); *carp-cdx1* is expressed in the tail bud and in the caudal neural keel (arrowhead). Furthermore, one of the paired strings of labeled lateral mesodermal cells is visible (arrow). **8'** Same embryo as Fig. 8, now seen from the dorsal side. Note the expression of *carp-cdx1* in the caudal part of the neural keel (arrowhead) and the lateral mesoderm (arrows). **8A** Section of embryo of Fig. 8, showing the most anterior and posterior parts of the embryo. The caudal part is labeled (tail bud). Only the yolk cell and the enveloping layer are negative. **8B** Section of embryo of Fig. 8, more anteriorly than the section of Fig. 8A. *Carp-cdx1* is expressed in the caudal neural keel (nk), in the dorsocaudal ectoderm (ec), in the most lateral mesodermal areas (lm) and in the endoderm (en). **9** Whole mount in situ hybridization of 24 hours old embryo, showing gene expression only in the caudal part of the tail.

for a protein containing the last three of the five conserved peptide sequences and a homeodomain with 80% amino acid identity to carp-cdx1. For another insect, *Bombyx*, this figure is even higher (Table 1).

Carp-cdx1 has many characteristics of *Antennapedia* class genes (Fig. 2). Genes of this class are defined (McGinnis and Krumlauf, 1992) by the sharing of 60% or more identity with the *Antennapedia* homeodomain (60%), the possession of identical sequences in α-helix III (1 substitution) and the presence of a Y-P-W-M motif 5′ to the homeodomain (Y-Q-W-M) (between brackets the figures for *carp-cdx1*).

Temporal and spatial expression of carp-cdx1

A weak expression of *carp-cdx1* was found 6 hours a.f. at the stage of 25% epiboly. This was shortly before the onset of gastrulation, which started after 50% of the yolk cell had been covered by the expanding blastoderm. The reaction product was present in the marginal area of the blastoderm, but was unequally distributed: a zone of 180° was relatively heavy labeled, and labeling faded out gradually from this area (Figs. 3, 3A). Within the marginal area the label was absent in the deepest blastomeres and the enveloping layer cells. As epiboly and gastrulation proceeded, the area of expression of *carp-cdx1* gradually increased. At early gastrulation (Fig. 4), epiblast cells in the marginal area were stained, but at the level of first involution, interpreted as the embryonic shield (Schmitz and Campos-Ortega, 1994), very weak or no label was found (Figs. 4, 4A, 4B, 4C). Just-involuted hypoblast cells were not labeled (Fig. 4C). At the end of epiboly, cells all around the remaining yolk plug showed expression of the gene (Fig. 5). Epiblast as well as hypoblast cells, which recently involuted along the rim of the blastoderm, were labeled at late gastrula stages (Figs. 5A, 5B, 5C), but the label was most intense in the cells of the epiblast. Earlier involuted cells were unlabeled.

At 10 hours, *carp cdx1* was expressed only in the caudal half of the embryo (Fig. 6). Fig. 7 shows the principle cell movements during gastrulation. Early involuting cells at the (dorsal) embryonic shield have an anterior fate, late involuting cells a posterior fate. As a result of convergent movements along the germ ring, a similar tendency exists for cells involuting laterally or ventrally.

At 14 hours a.f., the embryos had reached the 6-8 somite stage. The Kupffer's vesicle was present rostroventrally from the tail bud at this stage. As at 10 hours, only the caudal part of the embryo was labeled (Figs. 8, 8′). In the tail bud, all cells except those belonging to the enveloping layer showed *carp-cdx1* expression (Fig. 8A). Anteriorly, the tail bud extended over the caudal part of the yolk cell which was not labeled; Kupffer's vesicle did not express the gene either. Anteriorly from the Kupffer's vesicle, segmentation became visible. The most caudal somite appeared slightly positive, expression fading out anteriorly. Heavy labeling was also found in

the dorsal ectoderm and neurectoderm (neural keel; Fig. 8B), where labeling gradually weakened in more anterior parts and had disappeared at the latitude of the most anterior somites. A paired area with strong expression was located at the most lateral aspect of the mesoderm (Figs. 8', 8B). As in the neural keel, in the latter tissue a posterior to anterior gradient of label was found. The notochordal mesoderm did not express *carp-cdx1*. Between the notochord and the yolk cell, an axial string of labeled cells was found (Fig. 8B), which was interpreted as endoderm. These cells were found labeled relatively strongly in the caudal part of the trunk, and less extensively in more anterior areas.

At later stages, expression had retracted to the most caudal part of the embryo. At 24 hours, only the caudal tip of the tail showed expression of *carp-cdx1* (Fig. 9). At 48 hours a.f., the label could no longer be detected.

Discussion

Evolutionary and functional relationship between caudal homologs and genes residing in the HOM or Hox complexes

There is no doubt concerning the homology of *carp-cdx1* and a number of vertebrate *caudal*-like genes, seen in the degree of identity of their nuclear and amino acid sequences and the presence within the *carp-cdx1* nuclear sequence of five highly conserved areas specific for the *caudal* class of genes (Gamer and Wright, 1993). Several *caudal* homologs have been described in both *Xenopus* and mouse. Different expression patterns of different homologs do occur within a species. *Xcad-1* and *Xcad-2*, for example, are initially expressed solely in the dorsal lip (Blumberg et al., 1991), while *Xcad-3* (Northrop and Kimelman, 1994) shows an expression pattern much like that of *Zf-cad1* (Joly et al., 1992) and *carp-cdx1* (this paper). Similarly, mouse *Cdx-1* is expressed during somitogenesis in a number of tissues including the somites, in contrast to *Cdx-4* which, like *Xcad-3*, *Zf-cad1* and *carp-cdx1* is not expressed in the somites (Gamer and Wright, 1993). Therefore, a *caudal* class of genes with different functions may exist in vertebrate species.

Caudal and its homologs are neither localized within the *Drosophila HOM* (Mlodzik et al., 1985) nor in one of the four vertebrate *Hox* complexes (Frumkin et al., 1991), but reside elsewhere in the genome. Like the clustered genes, many other homeobox genes, including *carp-cdx1*, appear to be involved in axial patterning (McGinnis and Krumlauf, 1992; this paper). Furthermore, the *carp-cdx1* homeodomain as well as that of other *caudal* homologs (McGinnis and Krumlauf, 1992), appears very similar to that of the clustered *Hox* genes. *Carp-cdx1* needs only two amino acid substitutions (one in α-helix III and one in the Y-Q-W-M peptide) to meet the criteria for *Antennapedia* class homeodomains mentioned by McGinnis and Krumlauf

(1992).

An evolutionary tree of *Drosophila* homeodomains, as suggested by Scott et al. (1989), shows a close relationship between the *HOM* residing genes *labial* and *AbdB* (positioned at the most 3′ and 5′ ends of the *Antennapedia* and *Ultrabithorax* clusters, respectively) and the gene *caudal*. Furthermore, the three genes all appear to be involved in patterning the extreme anterior and posterior ends of the embryo. Possibly, one or more 5′ or 3′ located genes, among them the forerunner of *caudal*, have been separated from an ancient ancestral homeobox gene cluster during evolution.

Clustered homeobox genes have received much attention lately. It appears important, however, not to consider these genes separately from related non-clustered genes like *caudal* homologs. Interactions between caudal and *HOM/Hox* genes have been reported (see next paragraph). Furthermore, the *Hox* genes do not seem to depend on their specific location for functioning, as has been shown by the rescue of gene functions in mutants after the introduction of transgenes (Coletta et al., 1994).

Carp-cdx1 expression and the specification of the anteroposterior (a-p) axis

In the insects *Drosophila* and *Bombyx*, *caudal* is expressed as a maternal gene during the earliest stages of development, and as an embryonic gene from the syncytial blastoderm stage (Mlodzik et al., 1985; Xu et al., 1994). In vertebrates, however, *caudal* mRNAs only appear shortly before gastrulation, after the onset of embryonic gene expression. This also may hold for the carp, since increasing *carp-cdx1* expression is found only after the onset of epiboly, which may depend on zygotic gene function (Stroband et al., 1992). However, oocytes and early cleavage stages of carp have not yet been studied.

The expression pattern of *carp-cdx1* points to a function in a-p patterning of the embryo. The role of *caudal* homologs in axial specification may be of great importance. In *Drosophila*, *caudal* influences the expression of segmentation genes and indirectly downregulates certain *HOM* clustered genes (Mlodzik et al., 1990). In vertebrates, the *caudal* homologs might interact with *Hox* genes (McGinnis and Krumlauf, 1992). In the mouse, the *Cdx-1* expression pattern indicates a possible relationship to the expression of the clustered *Hox* genes: *Cdx-1* expression is downregulated from anterior to posterior areas after specific *Hox* genes are firstly expressed in these areas and, thus, disappears after axial positions are specified (Meyer and Gruss, 1993). Mouse *Cdx-4* is also expressed earlier than most *Hox* genes, and a function in the regulation of these genes is suggested (Gamer and Wright, 1993).

In amphibians, axial patterning is closely related to mesoderm induction and dorsalization, signaled by maternal growth factors early in development (Savard, 1992). Interestingly, in *Xenopus, Xcad3*, which is expressed in the marginal zone of the blastula, is induced by both basic fibroblastic growth

factor (bFGF) and activin in animal cap assays (Northrop and Kimelman, 1994). Furthermore, a dominant negative FGF-receptor downregulates *Xcad3*. It is possible that in carp, as in *Xenopus*, maternal growth factors from the vegetal part of the embryo (the yolk cell) induce gene expression in more animal parts (the blastoderm). These inductions might directly or indirectly lead to *carp-cdx1* expression as part of the a-p specification process. Recently, we started a study of gene expression in blastoderms which were isolated from the yolk cell. The first results suggest that from the stage of YSL (yolk syncytial layer) formation onwards blastoderms have the potency to express *carp-cdx1* without the presence of the yolk cell. If this is true, an eventual signal leading to *carp-cdx1* induction should be delivered to the blastoderm during cleavage, before YSL formation. In zebrafish, localized *no tail* expression was found in similar explants, suggesting the specification of dorsal axial mesoderm (Te Kronnie et al., 1994). This matter is the subject of further study.

Carp-cdx1 expression and the gastrulation process

The finding of increasing expression of *caudal* homologs from early to late epiboly stages in zebrafish (Joly et al., 1992) as well as in carp, suggests a function in the gastrulation process. In this respect it is interesting that in *C.elegans* the *caudal* homolog *pal-5* regulates *mab-5*, a gene playing a role in cell migration during gastrulation (Bürglin and Ruvkun, 1993). Homeobox gene expression in vertebrates also may contribute to regulation of cell migration (i.e. *goosecoid* in *Xenopus*; Niehrs et al., 1993). It is possible, that *carp-cdx1* expression leads to directional migration of cells with a posterior fate. Joly et al. (1992) found *Zf-cad1* expression in the region of the margin, but in early gastrulae a small rim of vegetalmost cells did not express the gene. According to the fate map of zebrafish, these early involuting cells may have an anterior fate in contrast to later involuting cells (Kimmel et al., 1990). In carp, similar unlabeled vegetalmost cells were not found. However, a relatively strong signal was present in ventral areas of the gastrula, which also may be correlated with a posterior fate. At early gastrula stages, involuted cells did not express *carp-cdx1*. This suggests that the cells lose expression during involution. Similar results were found by Joly et al. (1992; 1993) in somewhat later stages of gastrulation in zebrafish concerning *zf-cad* and *eve1*, a gene also expressed posteriorly. Interestingly, Joly et al. (1993) associate these findings with the existence in the rosy barb of dye coupling between the YSL and involuted blastomeres, as found in our group by Gevers and Timmermans (1991). The specification of early involuting cells might be changed from posterior to anterior. Further study is needed to see if blocking of dye coupling will influence *carp-cdx1* (and *eve1*) expression in intact embryos. At the end of epiboly, just-involuted cells are labeled, suggesting that downregulation during involution is a temporary process. Comparing results on carp and zebrafish one might conclude that the existence of a rim

of non-*cdx* expressing vegetalmost cells before gastrulation is not an essential element of cyprinid development, while the downregulation phenomenon appears essential.

Carp-cdx1 expression during segmentation

During somite formation, *carp-cdx1* expression is especially strong in the tail bud and in the caudal part of the neural keel. The same holds for *Zf-cad1* (Joly et al., 1992), *Xcad-3* (Northrop and Kimelman, 1994) and *Cdx-4* (Gamer and Wright, 1993). The posterior to anterior gradient of labeling in the neural keel suggests a function in patterning of the spinal cord. Gradually, expression is retracted in a caudal direction.

After formation of a new somite, most participating cells soon appear to lose *carp-cdx1* expression. It is likely, therefore, that *carp-cdx1* also has a function in somite formation. Not all mesodermal cells lose *carp-cdx1* expression after leaving the tail bud. A relatively small number of laterally localized mesodermal cells continue to express the gene. Their fate is unknown, but they could be forerunners of lateral plate mesoderm, judged from their location and since, in the mouse, *Cdx-4* is expressed in the lateral plate mesoderm (Gamer and Wright, 1993). Similar labeled cells have not been described for zebrafish. This difference may be due to the different staining technique: the use of radioactive label on sections may have resulted in a relatively low resolution.

Carp-cdx1 also appears to be expressed in the trunk endoderm. This also holds for a number of other vertebrate *caudal* homologs: *Zf-cad1* (Joly et al., 1992), *CHox-cad* (Frumkin et al., 1991), *Cdx-4* (Gamer and Wright, 1993) and a rat *caudal* homolog (Freund et al., 1992). A function in the formation of the gut epithelium is suggested. It is interesting that, in the mouse, *Cdx-2* is differentially expressed in the adult intestine (James and Kazenwadel, 1991), suggesting a role in cellular differentiation in this cell renewal system. Since the endoderm is the only axial structure, apart from the neural keel, showing *carp-cdx1* expression, it may be used as an endoderm marker in studies of early gut formation.

Materials and methods

Embryos

Carp (*Cyprinus carpio*) oocytes and sperm were obtained by stripping as described earlier (Stroband et al., 1992). All further handlings were carried out at 25°C. Only Cu^{++}-free tap water was used. After mixing 100 g eggs with 5 ml sperm, the latter was activated by the addition of 100 ml of an urea (0.5%) solution in tap water containing 0,2% NaCl and fertilization took place within 1 minute. Urea treatment was continued for 6 minutes. Afterwards, eggs were rinsed thoroughly in tap water for 24 minutes and

immersed in a tannic acid (0,35g/l) solution. Urea and tannic acid treatment were carried out in order to avoid adhesion of the eggs. After rinsing the eggs with tap water they were put into a flask through which a bottom to top water flow was established, again to avoid egg adhesion.

Construction of cDNA library

11.5 Hours a.f. the embryos had reached the early segmentation stage and the first somite pairs were formed. At that stage, embryos were transferred from the water column to 50 ml tubes and frozen using liquid nitrogen.

Total RNA was extracted using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). After homogenization of the embryos, chorions were removed by centrifugation (5-10 minutes, approximately 800 g, at 4°C), as they disturb the following phenol-chloroform extraction. Poly (A)+ RNA was selected with a PolyATtract mRNA isolation system (Promega, Madison, Wis, USA). A cDNA library was created in the Lambda ZAP II vector, using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, Calif., USA) and the Gigapack II Gold Packaging Extract (Stratagene) according to the manufacturer's instructions. The library contained approximately 1.3×10^6 independent plaque forming units (pfu) and was amplified once.

Cloning of homeobox containing genes

For the generation of a homeobox-specific probe a digoxygenin (Dig-[11]-dUTP; Boehringer, Mannheim)-labeled genomic fragment containing 120 basepairs was produced by PCR using the following primers derived from the α-helices 1 and 3 of the *Antennapedia* homeodomain (Fig. 2):

upstream primer: GGAGCTCGAGAAAGAATTTCAC
downstream primer: CCTCCTGTTCTGGAACCAGAT.

Genomic DNA (3 μg), isolated from early somite stage carp embryos, was used as a template. Taq DNA polymerase (Promega, Madison, Wis., USA) was used in combination with the supplier's buffer and a Mg^{2+} concentration of 1.5 mM. PCR was carried out for 50 cycles (denaturation at 94°C for 1 minute; annealing at 55°C for 1 minute; extension at 72°C for 2 minutes).

Of the above cDNA library, 10^6 pfu were plated and screened with the DIG-labeled probe. 26 Positive clones were rescreened. After PCR, using part of each plaque as a template and vector specific primers T3 and T7 (Stratagene), the lengths of the inserts of the clones could be estimated by gel electrophoresis. At least 10 different insert lengths, ranging from 0.9-3.0 kb were found. A 1.3 kb clone was sequenced and was used for the production of a probe for in situ hybridization.

DNA sequence analysis

Sequencing was carried out on both strands according to a dideoxy chain termination protocol using sequenase (USB, Cleveland, Ohio). T3 and T7 (vector specific) primers (Stratagene) were used as well as the above homeobox derived primers. Sequences were analyzed using the EMBL database and the Caos-Camm Fasta program. Sequence data will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under Accession Number X80668 C. carpio mRNA.

In situ hybridization

The method used is essentially that described by Westerfield (1993). Some modifications, however, have been adopted from Stachel et al. (1993) and Harland (1991). Embryos of stages between 3.5 hours (blastoderm) and 2 days (hatching) were used. In short the procedure was the following: after fixation in 4% paraformaldehyde at 0°C for 24 hours, the embryos were washed and mechanically dechorionated in PBS. Subsequently the embryos were transferred to 100% methanol and stored at -20°C.

After rehydration through a methanol series, the embryos were fixed again in paraformaldehyde (4%) in PBS at room temperature for 20 minutes, washed, and digested by proteinase K solution (10μg/ml) in PBS for 3-14 minutes. After washing again, a third fixation (20 minutes) was carried out. The embryos were incubated in prehybridization mix, containing 0.5% baker's yeast RNA in 50% formamide, 5xSSC, 50 μg/ml heparin and 0.1% Tween 20, for at least 2 hours at 60°C. Hybridization took place overnight at 60°C using the complete anti-sense DIG-labeled *carp-cdx1* RNA as a probe (sense RNA in controls), in a concentration of 0.05 ng/μl. The probe was generated by in vitro transcription of *carp-cdx1* cDNA in the presence of DIG-11-UTP (Boehringer, Mannheim) according to the instructions of the manufacturer. Washes (at 60°C) included 50% formamide in 2xSSCT (30 minutes), 2xSSCT (2x15 minutes) and 0.2xSSCT (2x30 minutes).

Staining of the embryos was performed overnight at 5°C, using the Dig nucleic acid detection kit (Boehringer, Mannheim) with alkaline phosphatase labeled anti-DIG Fab fragments at a concentration of 1:2000. For sectioning, stained embryos were embedded in paraffin. The colour of the reaction product is brown in whole mounts, but changes to blue (due to the dehydration process) in embryos used for sectioning.

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Chapter 3

Expression of Hoxb-1 during gastrulation, segmentation and early pharyngula stages of carp (Cyprinus carpio)

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Abstract

This report describes the cDNA sequence and embryonic RNA expression pattern of carp *Hoxb-1*. Carp *Hoxb-1* is a *labial*-like, homeobox-containing gene of the 3′ end of the *Hox* gene cluster. The expression pattern in carp is compared to that of homologs in other vertebrates. As holds for other *Hox* genes, carp *Hoxb-1* is expressed with highest intensity at a sharp anterior boundary, and expression fades out towards posterior. At later stages, gaps were found in the domain. The gene is expressed from late gastrulation onwards, first mainly in the hypoblast but later in all germ layers. Its most prominent expression area is rhombomere 4 (r4) of the hindbrain. Transcripts were also found in the neural tube, mesoderm (lateral, head and presomite), epidermis and neural crest. At 30 hours post fertilization, *Hoxb-1* was still expressed in r4, in the anterior trunk neural tube and in the branchial arches posterior to r4. *Hox* genes are thought to be involved in the specification of positional values along the embryonic anteroposterior axis, and *Hoxb-1* expression in r4 is supposed to be important for specifying the unique identity of this hindbrain segment. The conserved expression in r4 suggests that this is also true for carp *Hoxb-1*.

Introduction

Homeobox genes are widely assumed to play an important role in creating regional diversity along the embryonic a-p (anteroposterior) axis by providing positional values (see reviews by Krumlauf, 1994; Keynes and Krumlauf, 1994; Gehring et al., 1994). Genes belonging to this family encode proteins with a homeodomain, a DNA-binding motif of 60 aa (amino acids) that regulates the transcription of target genes (Gehring et al., 1994). After having been discovered in the *Bithorax* and *Antennapedia* gene complexes of the fruitfly, homeobox genes were found in many other species (reviewed in Ruddle et al., 1994). The vertebrate homologs of these genes are the *Hox* genes, present as four paralogous clusters: *Hoxa*, *Hoxb*, *Hoxc* and *Hoxd* (see Krumlauf, 1994), each cluster containing up to 13 genes.

The spatial and temporal order of expression of *Hox* genes along the anteroposterior axis of the embryo correlates with their order along the chromosome (colinearity rule, see Duboule and Morata, 1994). In the embryonic nervous system, a prominent site of expression of *Hox* genes, the anterior border of expression is sharp and lies in the spinal cord for the paralogous groups 5-13 (5′ parts of the clusters) and in the hindbrain for the groups 1-4 (3′ parts of the clusters) (Krumlauf et al., 1993).

Hox genes were also identified in fishes (Ericson et al., 1993; Molven et al., 1993; Alexandre et al., 1996; Marshall et al., 1994; Pöpperl et al., 1995), but of the anteriorly expressed *Hox* genes only the expression pattern

of *Hoxa-1* (Alexandre et al., 1996) has been described until now. In a search for *Antennapedia*-related homeobox genes (Stroband et al., 1995) in carp (*Cyprinus carpio*), a cyprinid teleost related to the zebrafish, we isolated *Hoxb-1*. This paper describes the sequence and expression pattern of the gene during gastrulation and segmentation of carp. As in chicken (Sundin et al., 1990; Sundin and Eichele, 1990), mouse (Murphy et al., 1989; Frohman et al., 1990) and *Xenopus* (Godsave et al., 1994), the anteriormost expression of the gene is found in a restricted region of the hindbrain. Thus, *Hoxb-1* might be a useful marker in future studies of hindbrain segmentation in fish.

Results

Sequence analysis of carp Hoxb-1

An *Antennapedia*-derived homeobox-specific probe was used to screen a carp early segmentation stage cDNA library (Stroband et al., 1995). One of the isolated clones had a length of 1852 bp (basepairs), with an open reading frame. It encoded a 316 aa protein related to *Drosophila labial*. The nucleotide and deduced aa sequences are shown in Fig. 1. The clone had a short 5′ UTR (untranslated region) defined by two potential translation initiation sites in frame with the homeodomain. The first start site was located in a suboptimal context (a C at position -3; Kozak, 1986) and was not a likely initiator of translation. In this sense it resembled the first start site of the mouse *Hox1.6* (*Hoxa-1*) gene (Larosa and Gudas, 1988). The second start site (5 aa downstream) in the carp gene contained an A at the -3 position, which created a favorable context for translation (Kozak, 1986). The 3′ UTR of 865 nucleotides showed a polyadenylation signal sequence (aataaa; Fig. 1) indicating that the 3′ UTR was complete.

Sequence analysis revealed that the clone was most closely related to the *labial* class of genes of the vertebrate *Hox* cluster, paralogous group 1. The deduced homeodomain of the carp protein had 92% identity to the *labial/Hox-1* homeodomain consensus sequence (Fig. 2A). The presence of a T at position 37, and an E instead of a Q at position 39 of the homeodomain (Fig. 2A) suggested that the carp gene belongs to the *Hoxb* complex (see Gehring et al., 1994). We therefore named this clone *Hoxb-1*, in accordance with the nomenclature suggested by Scott (1992). The homeodomain of carp *Hoxb-1* was highly similar to that of other species (Fig. 2A), especially chicken and *Xenopus* (97% aa identity). Mouse *Hoxb-1* and human *HOXB-1* homeodomains were both 88% identical to the carp *Hoxb-1* homeodomain. Outside the homeodomain, two other regions of high similarity between *Hoxb-1* of carp and other species existed. At the amino terminus (aa 1 to 22), fourteen aa were conserved between the fish gene product and the chicken, mouse and human proteins (Fig. 2B). Upstream of the

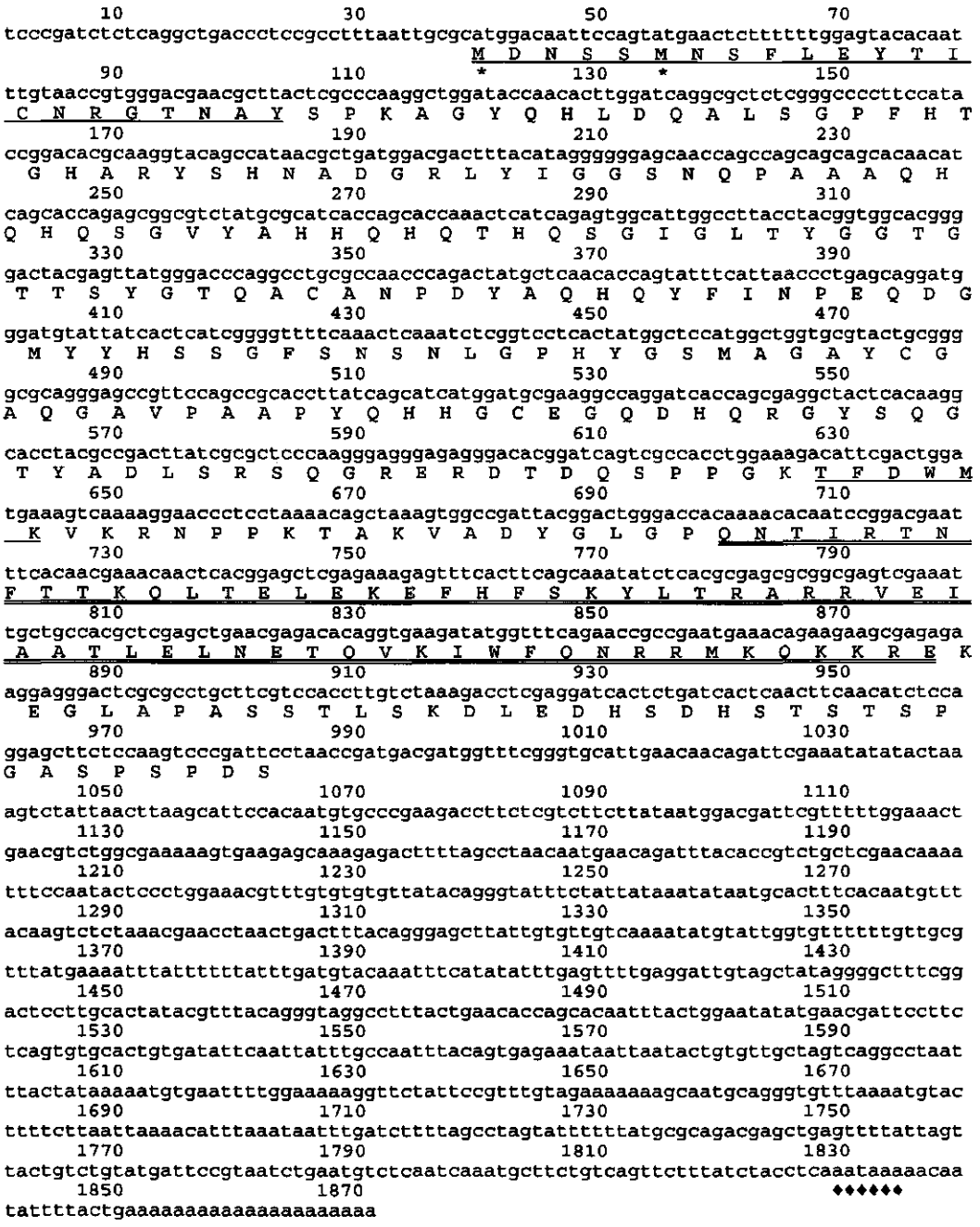


Fig. 1 Nucleotide and deduced amino acid sequence of carp *Hoxb-1*. The first underlined sequence represents the conserved N-terminus and the second underlined sequence is the conserved hexapeptide. The two translation initiation sites are marked by asterisks. The homeodomain is double underlined. The polyadenylation signal is indicated by ♦. The sequence is available from the EMBL database under Accession number X91079.

homeodomain, 100% identity was found in the *labial* class hexapeptide (TFDWMK: Bürglin, 1994; encoded by nucleotides 628 to 645 in carp) and the following 11 aa (Fig. 2C).

Early Hoxb-1 expression

During the process of gastrulation in carp and zebrafish, the blastoderm, originally located at the animal side of the yolk cell, thins and expands over the yolk cell by epiboly. After 50% epiboly, cells at the margin of the blastoderm involute and form the hypoblast layer. The outer cells form the epiblast. During completion of epiboly, involution continues while cells in the hypoblast and epiblast converge towards the midline thereby causing the embryonic axis to extend along the a-p axis. At the end of gastrulation, the epiblast layer is the future ectoderm. The hypoblast cells will give rise to the mesendoderm.

At early gastrulation stages, no RNA expression of *Hoxb-1* was observed. When 80% of the yolk was covered by the blastoderm (80% epiboly), weak *Hoxb-1* expression was detected near the equator of the egg, in two bands separated by the non-expressing midline. Since the YSL (Yolk Syncytial Layer) was heavily stained at many stages of carp development (Figs. 4E, 4F), the early expression (whole mount) could more easily be visualized in zebrafish (Fig. 3A). In sections of carp embryos we observed that the *Hoxb-1* expressing cells were found primarily in the hypoblast layer at this early stage (Fig. 4A). During the progression of gastrulation, the epiblast expression increased while the overall expression domain extended in a-p direction (Fig. 3B). At the bud stage (Kimmel et al., 1995), the *Hoxb-1* domain occupied the middle third region of the body axis. It showed a sharp anterior boundary and decreasing expression towards posterior. The most intense labeling at this stage was found in the epiblast, at the lateral borders of the expression domain (Fig. 3B).

Expression during segmentation

During carp segmentation, epiblast and hypoblast cells continue to accumulate in the midline, due to convergent extension movements as have been described for *Fundulus* (Trinkaus et al., 1992). As a result, the *Hoxb-1* expression was gradually gathered in a narrower region. Early in segmentation, the neural plate develops into the neural keel and subsequently into the neural rod by convergent movements of neural progenitor cells (described for zebrafish by Papan and Campos-Ortega, 1994). In the hypoblast layer, the notochord becomes distinct and somites form in the paraxial mesoderm in an anterior to posterior order. The first pair of somites arises at approximately 12 hours after fertilization at a position approximately halfway along the a-p axis in carp. The anterior half of the carp embryo is occupied by the developing head.

At the onset of segmentation, the *Hoxb-1* pattern was like that of the tail

A

labial/Hox 1 consensus	PNAIRTNFTT KQLTELEKEF HFNKYLTRAR RVETAATLQL NETQVKIWFQ NRRMKQKKRE
carp *Hoxb-1*	Q-T------- ---------- --S------- ---I----E- ---------- ----------
Xenopus Hoxb-1	Q-I------- ---------- ---------- ---I----E- ---------- ----------
chicken *Hoxb-1*	--T------- ---------- ---------- ---I----E- ---------- ----------
mouse *Hoxb-1*	-GGL------ R--------- ------S--- ---I----E- ---------- ----------
human *Hoxb-1*	-SGL------ R--------- ------S--- ---I----E- ---------- ----------

B

carp *Hoxb-1*	MDNSSMNSFL EYTICNRGTN AY
chicken *Hoxb-1*	---TR----- --A------G --
mouse *Hoxb-1*	--YNR-S--- --PL----PS --
human *Hoxb-1*	--YNR----- --PL----PS --

C

carp *Hoxb-1*	TFDWMKVKRN PPKTAKV
chicken *Hoxb-1*	---------- -------
mouse *Hoxb-1*	---------- -------
human *Hoxb-1*	---------- -------

Fig. 2 Comparison of conserved regions in Hoxb-1 sequences. **A** Comparison of the deduced homeodomain sequences of Hoxb-1 from carp, *Xenopus* (Dekker et al., 1992), chicken (Sundin et al., 1990), mouse (Frohman et al., 1990), human (Acampora et al., 1989), and the *labial/Hox-1* homeodomain consensus sequence (Gehring et al., 1994). **B** Comparison of the deduced amino-terminal regions of carp, chicken, mouse and human Hoxb-1. **C** Comparison of the deduced hexapeptide regions of carp, chicken, mouse and human *Hoxb-1*.

bud stage, except the anteriormost expression. At the anterior border of the *Hoxb-1* domain, intensely stained epiblast cells formed a transverse band (Fig. 3C). The band was situated at the level of the future hindbrain and it is supposed that cells in the band are probably neural progenitor cells based on the localization of these cells in zebrafish (Schmitz et al., 1993). In other vertebrates, *Hoxb-1* is expressed in r4 (rhombomere 4) (see references in the discussion). To confirm that this is also true for the carp, we performed double ISHs (in situ hybridizations) with zebrafish *Krox-20*, which is expressed in r3 and r5 (Oxtoby and Jowett, 1993). From the 1 somite stage up to late segmentation (Fig. 3F), *Hoxb-1* was expressed between the *Krox-20* r3 and r5 bands, as expected.

At the 8 somite stage, the dorsalmost cells in r4 appeared negative (Fig. 4B) whilst a small area of expression was present in the dorsalmost part of r3 (Figs. 3D, 4B). By the 15 somite stage, this latter expression had disappeared (compare Figs. 3D and 3E, 4B and 4F). The distribution of transcripts in r4 was homogeneous at the 8 somite stage, but at 15 somites the dorsal part of r4 expressed *Hoxb-1* at a higher level than ventral areas (Fig. 4E), including the floor plate. Ventrally, only a paired laterally located group of cells was intensely stained (Fig. 4E). This group may represent developing primary neurons, which are found at this position in zebrafish (Hanneman et al., 1988). Dorsolateral to the neural rod in r4, expression was found in a group of cells resembling migrating neural crest cells (Fig. 4E). Posterior to r4, the neural rod in the hindbrain did not express *Hoxb-1* at the stages of 8 and 15 somites (Figs. 4B, 4C, 4F). In the trunk the neural rod contained only a weak signal at the 8 somite stage, but at 15 somites this expression was more intense (Fig. 4F). At the level of the first somite (hindbrain-spinal cord transition), it had a sharp anterior boundary and faded out towards the tail. As in r4, the transcripts in the trunk neural rod were distributed in a dorsoventral gradient (not shown). Furthermore, at regular intervals along the a-p axis, as far anterior as r4, cells at lateral positions in the neural rod were intensely stained (Fig. 4F). These cells might be neurons of the dorsal longitudinal tract (DLT), as described for zebrafish by Ross et al. (1992). In some sections of the trunk at the 15 somite stage, labeled groups of cells were observed between the somites and the dorsal ectoderm. They could be neural crest cells (see Raible et al., 1992).

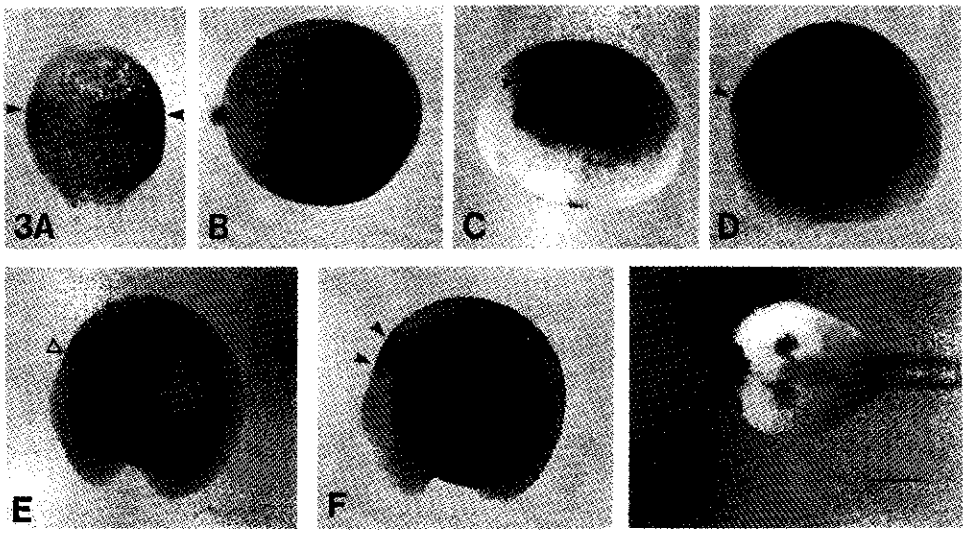
At 30 hours p.f. (post fertilization) the *Hoxb-1* expression in neural tissue was mainly restricted to r4 and the neural tube in the anterior trunk (Fig. 3G). A dorsoventral gradient could still be observed (not shown). At 52 hours p.f., faint labeling was detected only in r4, after prolonged staining.

Expression of Hoxb-1 outside the CNS

Expression of *Hoxb-1* was not confined to neural tissue, but appeared in cells of all germ layers. The strong expression in lateral areas, already seen at the bud stage (Fig. 3B), persisted through segmentation. As cells

Fig. 3 Expression of carp *Hoxb-1* during late gastrulation, segmentation and early pharyngula stages in whole mount in situ hybridizations (ISH) in carp (**B-G**) and zebrafish (**A**). Scale bar is 275 μm. **A** Late gastrulation stage zebrafish embryo with the animal pole to the top. The black arrowheads point at the expression on either side of the midline (open arrowhead points at the midline). **B** 0 somite stage carp, dorsal view with anterior to the left. The arrowheads point at the lateral stripes in the epiblast. **C** 1 pair of somites, dorsolateral view with anterior to the left. The arrowhead points at the transverse band of expression in the epiblast. **D** 8 pairs of somites, lateral view with anterior on the left side. Dorsalmost cells in r3 are stained (arrowhead). **E** 15 pairs of somites, lateral view with anterior on the left. Dorsal cells in r3 no longer express *Hoxb-1* (open arrowhead). **F** As **E**, double ISH of carp *Hoxb-1* (brown staining) in r4, and zebrafish *Krox-20* (orange staining) in r3 and r5 (arrowheads). **G** Dorsal view of carp embryo at 30 hours p.f. The black arrowhead points at the r4 expression. The open arrowhead points at the bilaterally symmetrical structures lateral of the neural tube.

Fig. 4 Expression of *Hoxb-1* in sectioned carp embryos at gastrulation and segmentation stages. **A** Horizontal section at the stage of 80-90% epiboly, dorsal is to the top left. The arrowheads point at the expression in the hypoblast. The midline (open arrowhead) does not express *Hoxb-1*. **B** Sagittal section through the neural rod at the 8 somite stage, with anterior to the left. The transverse band of expression is located in the future rhombomere 4 (r4). The black arrowhead points at the expression in the dorsalmost cells in r3. The hindbrain posterior to the future r4 does not express *Hoxb-1* (open arrowheads). **C-D** Cross sections at the 8 somite stage. **C** Expression at the level of the hindbrain, posterior to r4. The arrowheads point at the expression in the lateral head mesoderm and epidermis, corresponding to the stripes on both sides of the neural rod in the whole mount expression pattern. **D** Section at the level of the anterior trunk. The arrows point at the expression in the lateral mesendoderm, the arrowheads indicate the labeled epidermis, s = somite. **E** Cross section through rhombomere 4 at the stage of 15 somites. The black arrowheads point at the ventrolateral mesoderm; note that the YSL is also stained, but aspecifically. The white arrowheads point at the groups of cells resembling developing primary neurons, the open arrowhead indicates the expression in cells resembling the migrating cells of the neural crest, n = notochord. **F** Sagittal section through the lateral side of the neural tube at the stage of 15 somites. The dorsalmost cells in r3 no longer express *Hoxb-1* (open arrowhead). Intensely stained cells might be neurons of the dorsal longitudinal tract (arrows point at some of these cells). **G** Cross section at 30 hours p.f. at caudal position of the otic vesicle. *Hoxb-1* expression is found in leaf-like structures at ventrolateral positions (arrowheads). Scale bar is 65 μm in E, 99 μm in **C**, **D**, **G**, and 128 μm in **A**, **B**, **F**.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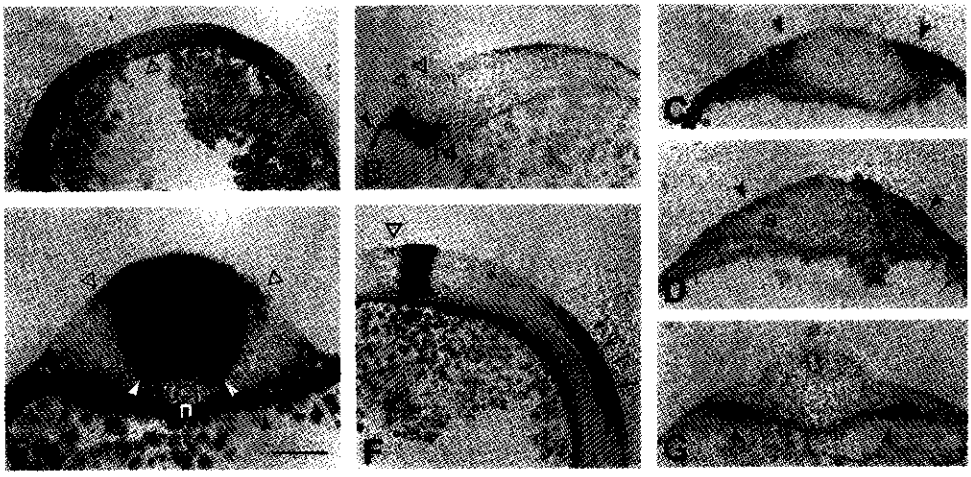
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converged to the body axis, the expression in lateral areas was concentrated in a prominent stripe on both sides of the neural tissue (not shown). In the trunk at 8 somites, this stripe was composed of expression in lateral mesendoderm and in the epidermis above the somites (Fig. 4D). The somites were largely negative, but the presomitic mesoderm contained the signal (not shown). At the level of the posterior hindbrain, *Hoxb-1* expressing cells were present in head mesoderm and epidermis (Fig. 4C). At this stage, the expression pattern showed a gap between the level of r4 and the posteriormost part of the hindbrain (not shown). Transcripts had reappeared again in this region by the 15 somite stage. At the level of r4 at the 8 somite stage, the *Hoxb-1* signal was present in lateral mesoderm, and by 15 somites in the ventrolateral mesendoderm (Fig. 4E).

At 30 hours p.f. only two symmetrical structures posterior to the otic vesicle represented expression outside the CNS (central nervous system) (Fig. 3G); sections revealed that these structures were ventrolaterally localized (Fig. 4G). They may correspond to outpocketings of the pharyngeal endoderm in the pharyngeal arches, as described for zebrafish by Schilling and Kimmel (1994). At 52 hours p.f. no expression could be detected outside the CNS.

Discussion

Identification of carp Hoxb-1

Carp *Hoxb-1* could be identified because of the high degree of identity of its nucleotide and amino acid sequences to those of *Hoxb-1* of other vertebrates (mouse *Hox-2.9*: Murphy et al., 1989; Wilkinson et al., 1989; chicken *Ghox-lab*: Sundin et al., 1990; *Xenopus Hoxb-1*: Godsave et al., 1994; man *HOXB1:* Acampora et al., 1989), especially to the homeodomains of chicken and *Xenopus* (97%). The presence of two other highly conserved areas further confirmed the homology to *Hoxb-1* genes of the other vertebrates. Furthermore, in all vertebrates, including carp, *Hoxb-1* has its anterior border of expression in the hindbrain, just like other members of the paralogous groups 1-4 (Krumlauf, 1994), and is specifically expressed in r4 (see references elsewhere in discussion), suggesting analogous functions as well. The *Hoxb-1* gene is a paralog of the *Drosophila labial* gene (Mlodzik et al., 1988) and belongs to the paralog 1 group (*Hoxa-1, Hoxb-1, Hoxd-1*) of the vertebrate clustered homeobox genes (*Hox* genes). The *Hoxb-1* gene is located at the 3′ end of the *Hoxb* cluster, and is among the first *Hox* genes to be transcribed in developing embryos (Krumlauf, 1994). So far, for fishes limited information is available about *Hox* genes with anterior borders of expression in the hindbrain. The sequence and expression pattern of zebrafish *Hoxa-1*, a *labial* paralog like *Hoxb-1*, were recently described in a study by Alexandre et al. (1996). In addition, 3′ and 5′ regions of the pufferfish

Hoxb-1 gene were identified, that are involved in the establishment of the expression pattern of the gene (Marshall et al., 1994; Pöpperl et al., 1995). This paper is the first to report the complete cDNA sequence and the RNA expression pattern of *Hoxb-1* in fish.

Early Hoxb-1 expression

Carp *Hoxb-1* transcripts were not detected until approximately 80% epiboly, which is slightly later than zebrafish *Hoxa-1*, expressed from 50% epiboly (Alexandre et al., 1996). At this early stage, *Hoxb-1* was found mainly in the hypoblast. During completion of gastrulation, the transcription in the epiblast increased. At the end of epiboly both the hypoblast and the epiblast expressed the gene, and in that respect the pattern is comparable to those of the mouse and chicken where *Hoxb-1* was expressed in the primitive streak in the epiblast as well as in the mesoderm (Murphy et al., 1989; Frohman et al., 1990; Murphy and Hill, 1991; Sundin et al., 1990). At this stage, the anteriormost boundary of expression in the epiblast was at the same a-p level as that in the hypoblast, similar to mouse and chicken (Frohman et al., 1990; Murphy and Hill, 1991; Sundin et al., 1990). The intensely labeled cells at the lateral borders of the expression domain at the tail bud stage might correspond to progenitor cells of the neural crest and epidermis, given the position of these progenitors in zebrafish (see Schmitz et al., 1993). For mouse, chicken and *Xenopus* this strong lateral expression was not reported.

Expression in neural tissue

A prominent and well-described feature of the vertebrate *Hoxb-1* pattern is the expression in (the future) r4 (mouse: Murphy et al., 1989; Wilkinson et al., 1989; Frohman et al., 1990; Murphy and Hill, 1991; chicken: Sundin et al., 1990; Maden et al., 1991; Guthrie et al., 1992; *Xenopus*: Godsave et al., 1994; man: Acampora et al., 1989). This characteristic r4-expression is also conserved in carp, as was demonstrated by the double ISH using *Krox-20*, which is expressed in r3 and r5 (Oxtoby and Jowett, 1993). Having been established at the onset of somitic segmentation at the anteriormost border of the expression region, the r4-signal persisted until after 30 hours p.f. After the first appearance of the r4-band, the expression in other regions of the presumptive neural tube was low, until the 15 somite stage when *Hoxb-1* was present in the trunk anterior neural rod. A separation between the r4-signal and more posterior CNS expression was previously described for chicken *Hoxb-1* (Sundin and Eichele, 1990; Maden et al., 1991). This separation makes *Hoxb-1* a useful marker for studies of segmentation of this part of the hindbrain. As was reported for the mouse (Frohman et al., 1990), the *Hoxb-1* expression in the future r4 of carp was already established before the segments of the hindbrain were recognized morphologically (for zebrafish at 17 hours; Hanneman et al., 1988). The exact role of this and other *Hox*

genes in the segmentation process is not clear yet. It has been proposed that *Hox* genes may play a role in the specification of the segment phenotype (Keynes and Krumlauf, 1994). The *Hoxb-1* gene may be involved in specifying the unique identity of r4 (Guthrie et al., 1992; Murphy and Hill, 1991; Murphy et al., 1989).

Recent work shed light on the regulatory mechanism behind the persistent expression in r4 (Studer et al., 1994; Ogura and Evans, 1995; Pöpperl et al., 1995). A conserved regulatory region of the *Hoxb-1* gene is supposed to control the maintenance of the r4-expression through a positive and direct autoregulatory feedback mechanism. In the light of the conserved nature of the mechanism and the conservation of the *Hoxb-1* r4-expression (Pöpperl et al., 1995), it is very likely that this regulatory mechanism also applies to carp.

In the trunk neural tube at the 15 somite stage, the anterior limit of expression was at the level of the first pair of somites, with expression decreasing towards the posterior end of the embryo. In mouse and chicken, the trunk neural tube also showed transcription of the gene (Murphy et al., 1989; Murphy and Hill, 1991; Wilkinson et al., 1989; Sundin et al., 1990). At the 15 somite stage and at 30 hours p.f. we observed a dorsoventral distribution of transcripts showing highest expression in dorsal regions of the neural tube. This dorsoventral distribution of transcripts was detected in r4 as well. Such a gradient of *Hoxb-1* expression in neural tissue was also reported for day 9-10 mouse embryos (Murphy and Hill, 1991), but not for *Xenopus* and chicken. Dorsolateral to the neural tube in r4 at the 15 somite stage, carp *Hoxb-1* mRNA was found in the migrating cells of the neural crest. In chicken (Sundin and Eichele, 1990) and mouse (Murphy and Hill, 1991), the neural crest cells derived from r4 also express the gene. In spite of the high level of dorsal expression, the dorsalmost cells in carp r4 did not express *Hoxb-1*, which resembled the mouse expression pattern, that showed no transcripts in a dorsal structure called the roof plate (Murphy et al., 1989). In carp, the ventralmost cells (including the floor plate) contained no or a low amount of *Hoxb-1* transcripts. Similarly, the mouse floor plate did not express *Hoxb-1* (Murphy and Hill, 1991). Not corresponding to this gradient however, groups of cells at ventrolateral positions in r4 (probably developing primary neurons) were intensely stained at the 15 somite stage. This aberration from the dorsoventral pattern was only described for carp.

The dorsoventral expression pattern of carp *Hoxb-1* in the neural tube might be attributed to ventralizing and dorsalizing signals, which originate from the axial mesoderm (Strähle and Blader, 1994) and the non-neural ectoderm (Dickinson et al., 1995), respectively. Mouse *Hoxb* genes display a dorsoventral distribution of transcripts in the neural tube as well (*Hox-2*; Graham et al., 1991). The genes displayed dynamic dorsoventral patterns. Between 12.5 and 14 days post coitum, the expression of all genes was restricted to dorsal positions in the neural tube, similar to carp *Hoxb-1*. It was

suggested that the *Hoxb* expression patterns mirror the birth of major classes of neurons in the CNS, and that the *Hoxb* genes provide each successive class of newly-born neurons with anteroposterior positional information.

Expression outside the CNS

Besides the expression in the CNS, carp *Hoxb-1* was also abundantly expressed in cells of the mesendoderm, epidermis and probably the neural crest. The overall expression pattern in these tissues changed as a result of convergence movements. The expression was initially found in a broad area on the dorsal side of the embryo but narrowed as the embryonic cells concentrated around the axis. A similarly changing expression pattern as a result of cell migrations was described for the zebrafish *engrailed-2* gene (Fjose et al., 1992). In addition, the distribution of *Hoxb-1* mRNA changed as cells differentiated, for example the signal had largely disappeared in the somitic mesoderm.

In general, mouse and chicken *Hoxb-1* transcripts were found in tissues comparable to those in carp, for example in lateral plate and presomite mesoderm, head mesoderm and ectoderm (Murphy and Hill, 1991; Frohman et al., 1990). In contrast to this, Godsave et al. (1994) reported for *Xenopus* that *Hoxb-1* was not expressed in mesodermal tissues at any of the developmental stages examined. In this respect *Xenopus* expression resembles that of the ascidian *Halocynthia roretzi Hox-1* gene (*HrHox-1*) which is expressed exclusively in ectodermal tissues in early embryogenesis (Katsuyama et al., 1995).

Another difference in expression was observed for the gut. In mouse and chicken the gene was expressed in tissues of the gut (Murphy and Hill, 1991; Sundin et al., 1990). Although we observed staining in mesendoderm cells in carp at the 8 somite stage, we detected no transcripts in gut-tissue at 25 hours p.f.

In addition, the distribution of transcripts in the branchial arches of mouse was studied in detail (Frohman et al., 1990). In 30 hours carp and zebrafish embryos we observed *Hoxb-1* in a structure resembling the outpocketing of pharyngeal endoderm in a branchial arch (described by Schilling and Kimmel 1994).

We conclude that carp *Hoxb-1* can be positively identified by comparing its sequence and expression pattern to those of vertebrate homologs. The largely identical expression patterns suggest similar functions for *Hoxb-1* in the various vertebrate classes, especially in the specification of the fourth rhombomere of the hindbrain, and in the dorsoventral patterning of part of the central nervous system.

Materials and methods

Embryos

The stripping of carp in order to obtain oocytes and sperm and the subsequent in vitro fertilization and culturing of embryos was performed as described by Stroband et al. (1995).

Cloning of carp Hoxb-1

Carp *Hoxb-1* was isolated from a carp early segmentation stage cDNA library after screening with an *Antennapedia*-derived homeobox-specific probe. The production of this probe and the subsequent screening of the library were described in Stroband et al. (1995).

DNA sequence analysis

Carp *Hoxb-1* was sequenced using a dideoxy chain termination protocol using sequenase (USB, Cleveland, Ohio). Both strands were sequenced using vectorspecific T3 and T7 primers (Stratagene, La Jolla, CA) and clone-specific primers. Similarity searches were carried out with the Fasta programs (Pearson and Lipman, 1988). The sequence described in this paper is available from the EMBL database under Accession number X91079.

In Situ Hybridization

Plasmid DNA was digested with EcoRI, phenol-chloroform extracted and subsequently precipitated with ethanol and used as template for the transcription reaction. Dig-labeled antisense RNA probes were synthesized with T7 polymerase using the DIG RNA-labeling kit (Boehringer Mannheim (BM), Germany). The carp *Hoxb-1* probe was diluted 200 times for in situ hybridization reactions, and the probe derived from the zebrafish *Krox-20* plasmid (generously provided by G. M. Kelly, University of Western Ontario, Canada) was diluted 4000 times. The in situ hybridizations were carried out as described earlier (Stroband et al., 1995), with the following modifications; the length of the proteinase K (10 μg/ml) treatment was adjusted for each new batch of proteinase K. The (pre)-hybridization solution contained 50% formamide, 2 x SSC, 2% blocking solution (BM), 0.1% Tween-20, 5 mg/ml yeast torula RNA (BM) and 50 μg/ml heparin. The antibody incubation lasted for 3 hours up to overnight.

In the double in situ hybridization, the hybridization solution contained the carp *Hoxb-1* (DIG-labeled) and the zebrafish *Krox-20* (fluorescein-labeled) probes. The detection of the *Krox-20* transcripts with the alkaline phosphatase conjugated anti-fluorescein antibody (BM), using fast red (BM) as a substrate, was stopped by a 15 minutes incubation in 0.1 M glycin Ph 2.2 in aquadest. Next, the embryos were washed 4 times in PBST (5 minutes each wash), preincubated in PBST with 1% blocking stock (PBST-BL)(BM) for 3 hours and incubated in 1:2000 alkaline phosphatase conjugated anti-DIG

Fab fragments (BM) in PBST-BL. After washing, the *Hoxb-1* transcripts were detected using NBT/X-phosphate (BM) as a substrate.

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Chapter 4

Isolation of carp cDNA clones, representing developmentally-regulated genes, using a subtractive-hybridization strategy

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Abstract

A subtractive-hybridization technique, combined with differential screenings and subsequent whole mount in situ hybridization (ISH) reactions, was used to isolate novel cDNA clones representing developmentally-regulated genes of carp. Small-scale differential screenings of an oocyte and a segmentation stage cDNA library using oocyte-specific and segmentation stage-specific enriched probes, yielded 75 positive clones. ISH screening showed that 65% (15) of the oocyte stage clones and 50% (26) of the segmentation stage clones were indeed stage-specific. Partial sequence analysis suggests that approximately 65% of the 41 stage-specific clones represent novel genes. In addition, an *Otx1* clone was isolated. Two novel clones and the *Otx1* clone are of special interest for developmental studies. The clones represent genes that are locally expressed during embryonic development. The expression patterns of *Otx1* and one of the novel clones suggest functions in specification of the anteroposterior (a-p) axis. The three clones provide molecular markers for the study of gastrulation and the patterning of the a-p axis in teleosts.

Introduction

We applied a subtractive hybridization strategy to search for novel, stage-specifically expressed genes involved in early cyprinid development. Novel genes could provide new insights into developmental mechanisms or refine existing hypotheses. For this study we chose the carp (*Cyprinus carpio*), a cyprinid teleost related to the zebrafish. Despite the relatively large evolutionary distance between carp and zebrafish (Meyer et al., 1993), their development is strikingly similar (for carp development see Neudecker, 1976).

Two developmental stages were selected for subtraction, both related to topical developmental issues, namely the oocyte and the early segmentation stages. Firstly, the mechanism for the determination of mesoderm in fish is largely unknown. In *Xenopus*, the maternal Vg1 RNA is vegetally localized and appears to have a function in mesodermal and endodermal pattern formation (Weeks and Melton, 1987). On the contrary, the homologous zebrafish DVR-1 transcript is distributed equally among all blastomeres (Helde and Grunwald, 1993). Therefore, it is interesting to study the localization of other maternal gene products in the fish egg, so we chose the oocyte stage for subtraction.

Secondly, the mechanism for determining endoderm has still not been clarified. Prospective endodermal cells are the first cells to involute (Warga and Kimmel, 1990), but the molecular mechanisms underlying the timing of involution, and the eventual commitment to the endodermal fate are

unknown. We therefore chose the early segmentation stage as a second stage for subtraction. By identifying stage-specific transcripts, we hope to provide tools for future studies dealing with these interesting topics.

The essence of the subtractive hybridization strategy applied here is the use of enriched probes to screen cDNA libraries of two distinct developmental stages. Enriched probes were made using a subtractive-hybridization technique described by Wang and Brown (1991) and the screenings of libraries were performed as differential plaque hybridization (eg. Almendral et al., 1988). Here, we present the first results of applying the strategy and we describe the ISH patterns of three clones that represent genes with localized expression during development.

Results

Plaques that were positive on the plus filter and negative on the minus filter in the differential screening were considered supposedly stage-specific. We selected 23 clones from the oocyte library and 52 clones from the early-segmentation library. Whole mount in situ hybridization (ISH) reactions were performed to confirm the stage-specificity and to provide information on the localization of gene expression. These results are summarized in Table 1. Non-specific binding of the α-DIG antibody to the yolk cell was observed in segmentation stage embryos and sometimes in cleavage stage embryos. Specific binding revealed that 65% of the isolated oocyte clones and 50% of the early-segmentation clones were indeed stage-specific. A number of clones appeared of importance from a developmental biology viewpoint because of their localized ISH pattern. Sequence analysis revealed novel sequences, besides clones that could be identified as known genes (listed in Table 2).

ISH and sequence analysis

Of the 23 oocyte-derived clones, 15 clones recognized RNAs that were mainly present during the cleavage stages (Table 1). Eleven probes represented genes that were locally expressed early in development; the RNAs involved disappeared gradually after late cleavage stages. Eight of these clones were homologous to 16s rRNA (Table 2) and appeared to hybridize in the enveloping layer at 50% epiboly. The three remaining early stage-specific clones (1-9, 1-22, 1-27) represented one novel gene. The RNA detected by these clones was still present at the 50% epiboly stage when the highest concentration was located in the germ ring and the embryonic shield (Fig. 1). During segmentation stages, the gene was no longer expressed (Fig. 1). Clone 1-22 was completely sequenced and as yet has shown no resemblance to any known sequence.

Twenty-six early-segmentation stage-derived clones represented genes that

Table 1 In situ hybridization patterns

clone origin & number	length (kb)	hybridization of clone cs	lb	e	1s	8s	stage-specific?
oocyte stage:							
1-9 1-22 1-27	1.3-1.9	+	+	+a	-	-	yes
1-10	2.0	-	nd	-	nd	-	no
1-11	1.6	-	-	-	-	-	no
1-12	1.4	+	+	+	+	+	no
1-13*	1.2	+	+	+	+	+	no
1-14 1-15 1-16 1-17*	0.5-0.9	+	+	+a	-	-	yes
1-19 1-21 1-24 1-25*	0.4-1.0	+	+	+a	-	-	yes
1-18	0.5	-	-	-	-	nd	no
1-20	1.6	+	+	+	-	-	yes
1-23	1.4	+	+	±	-	-	yes
1-26	1.4	+	+	-	-	-	yes
1-28	1.6	-	-	-	-	nd	no
1-29	>2.0	-	nd	+	±	nd	yes / incorrect
1-30	1.4	+	+	+	-	-	yes
1-31	0.7	-	-	-	-	-	no
segmentation stage:							
3-1 3-31 3-52	1.0-1.6	-	+	+	+a	+a	yes
3-2*	0.8	+	+	+	+	+	no
3-3	>2.0	-	+	+	+	+	yes
3-4	1.3	-	+a	+a	+a	nd	yes
3-5 3-21 3-27	0.8-1.1	-	+a	+a	+a	+a	yes
3-6*	1.6	-	+a	+a	+a	+a	yes
3-7	0.2	nd	nd	nd	nd	nd	nd
3-8	0.8	+	+	+	+a	+a	no
3-9 3-10*	2.0	-	+a	+a	+a	+a	yes
3-11 3-25*	0.8-0.9	+	+	+	+	+	no
3-12*	0.7	+	+	+	+	+a	no
3-13*	>2.0	±?	±	-?	-	-	yes / incorrect
3-15*	1.3	+	+	+	+	+	no
3-16	>2.0	-	-	-	±a	±a	yes

clone origin & number	length (kb)	hybridization of clone cs	lb	e	1s	8s	stage-specific?
segmentation stage (cont'd):							
3-17	>2.0	+	+	+	+	+	no
3-18	0.6	+	+	+	+a	+a	no
3-19	1.2	-	-	+	+a	+a	yes
3-20*	1.0	-	±	+	+a	+a	yes
3-22	1.6	+	+	+	+	+	no
3-23*	0.8	-	±	+	+	+a	yes
3-24	1.5	+	+	+	+a	+	no
3-26	1.4	+	+	+	+	+	no
3-28	1.0	-	+a	+a	+a	+a	yes
3-29	1.1	-	+	+	+a	+a	yes
3-30 3-33 3-61*	0.8-1.2	+	+	+	+a	+a	no
3-32*	1.6	nd	nd	nd	nd	nd	nd
3-34*	1.4	-	-	-	-	-	no
3-35	0.6	-	+	+	+	+	yes
3-36	1.0	-	+	+	+	+	yes
3-39*	1.0	-	+a	+a	+a	+a	yes
3-40	1.5	+	+	+	+	+	no
3-41	1.3	-	±	+	+	+	yes
3-43	1.4	-	±	±	-	-	yes / incorrect
3-44	1.3	-	±	±	-	-	yes / incorrect
3-45	0.8	-	+	+	+	+	yes
3-46	>3	-	+	+	+	-	yes
3-47	1.9	-	+	+	+	+	yes
3-48	>2.0	-	±	+	+a	+a	yes
3-49	0.6	+	+	+	+	+	no
3-50	0.6	-	+	+	+	+	yes
3-53	1.6	-	+	+	+	nd	yes
3-55*	0.9	+	+	+a	+a	+a	no
3-57	1.1	-	-	-	-	-	no
3-59*	1.4	+	+	+	+	+	no

Embryonic stages: cs = cleavage stages; lb = late blastula stages; e = 50% epiboly stage; 1s = approximately one pair of somites; 8s = approximately 8 pairs of somites. □: stage-specific hybridization is boxed. Abbreviations: a = localized hybridization; nd = not determined; * = identity of clone is described in Table 2. Clone-lengths are determined by gelelectrophoresis and are approximate (± 0.2 kb).

Table 2 Sequence analysis results of FASTA program searches (*AC* Accession Number of the GenEMBL databases)

Clone	Search Results
1-13	95% identity to a 169 bp segment of the cytochrome b coding region of *C. carpio* complete mitochondrial genome DNA. AC X61010; Chang et al. 1994.
1-14, -15, -16, -17, -19, -21, -24, -25	93-97% identity to a 133-161 bp segment of the 16s rRNA coding region of *C. carpio* complete mitochondrial genome DNA. AC X61010; Chang et al. 1994.
3-2	95% identity to a 91 bp segment of the cytochrome c oxidase subunit 1 coding region of *C. carpio* complete mitochondrial genome DNA. AC X61010; Chang et al. 1994.
3-6	71% identity to a 69 bp region of *O. aries* KII-9 gene for hair type II keratin intermediate filament protein. AC X62509; Powell et al. 1992.
3-9, -10	74-77% identity to a 121-167 bp regions of *X. laevis* mRNAs for cytokeratin type II (XenCK55(5/6)). AC X14427; Fouquet et al. 1988.
3-11, -25	97-99% identity to an 85-205 bp segment of the cytochrome c oxidase subunit III coding region of *C. carpio* complete mitochondrial genome DNA. AC X61010; Chang et al. 1994.
3-12	79% identity to a 172 bp region of hamster mRNA for ribosomal protein S4. AC D11087; unpublished.
3-13	90% identity to 149 bp region of *B. rerio* translation elongation factor 1 (EF-1) alpha mRNA. AC X77689; Nordnes et al. 1994.
3-15	99% identity to a 134 bp segment of the NADH ubiquinone oxidoreductase subunit 2 coding region of *C. carpio* complete mitochondrial genome DNA. AC X61010; Chang et al. 1994.
3-20	84% identity to a 217 bp region of human ribosomal protein S4 (RPS4X) isoform mRNA. AC M58458; Fisher et al.,1990.
3-23	86% identity to a 145 bp region of mouse alpha-tubulin isotype M-alpha-3 mRNA. AC M13442; Villasante et al. 1986.
3-30, -33, -61	92-94% identity to a 126-185 bp segment of the cytochrome c oxidase subunit II coding region of *C. carpio* complete mitochondrial genome DNA. AC X61010; Chang et al. 1994.
3-32	91% identity to an 81 bp region of *C. auratus* intermediate filament protein type II keratin mRNA. AC M87773; Giordano et al. 1989.
3-34	in reverse orientation: 85% identity to a 163 bp region of *B. rerio* elongation factor 1 (EF-1) alpha mRNA. AC X77689; Nordnes et al. 1994.
3-39	95% identity to a 162 bp region of *C. auratus* keratin mRNA. AC L09743; Druger et al. 1994.
3-55	93% identity to a 138 bp region of *B. rerio* orthodenticle-related protein 1 (zOtx1) mRNA. AC U14591; Li et al. 1994 and Mori et al. 1994.
3-59	84% identity to a 196 bp region of *O. keta* mRNA for alpha-tubulin. AC X66973; Coe et al. 1992.

were expressed after late cleavage stages. Nine clones recognized RNAs that had an even distribution throughout the embryo, for example 3-36 (Table 1, Fig. 2). None of these clones have been identified so far. The other 17 probes hybridized to RNAs which were not homogeneously distributed in the embryo, for example clone 3-28 (Table 1, Fig. 3). At 50% epiboly, intense hybridization was found in the germ ring and the embryonic shield (Fig. 3). During segmentation stages, the staining was localized in the embryo in a decreasing gradient from posterior to anterior. Clone 3-28 was completely sequenced and no homology to any known gene has been detected yet. Among the other 16 clones, partial sequencing revealed 4 keratins, a ribosomal protein 4s and an alpha-tubulin (Table 2).

Six oocyte-derived and 6 segmentation stage-derived clones have yet to shown unambiguous hybridization patterns in ISHs. All clones were unknown except for two early-segmentation clones which were identified as elongation factors (Table 2).

Twenty clones hybridized in all embryonic stages examined and were thus not considered stage-specific. Among these were a cytochrome b, six cytochrome c oxidase subunits, a ribosomal protein s4, an alpha-tubulin and an NADH ubiquinone oxidoreductase subunit. Twelve clones hybridized to RNAs that occurred homogeneously in the embryo, often also in the yolk syncytial layer (YSL), but not in the yolk. Other clones represented genes with local differences in expression, for example 3-55 (Table 1, Fig. 4). At approximately 50-60% epiboly, the latter probe detected RNAs all over the blastoderm with the highest hybridization signal in the involuted cells of the embryonic shield (Fig. 4). In the early-segmentation stage (one pair of somites), the highest concentration of the RNA was found in a small region behind the eyes. In later segmentation stages, the intensity of expression decreased, but remained in the brain (Fig. 4). Sequence analysis revealed that the clone is similar to orthodenticle-related genes of other vertebrates, especially those described for the zebrafish (Table 2).

Discussion

Subtraction strategy

Many different strategies have been described for the isolation of genes regulating embryonic development. A technique for isolating genes that show temporary expression is +/- (plus-minus) screening. In a +/- screening that we performed (results not shown), many clones displayed some hybridization to both the + and the - probes and the lightly stained clones (probably resembling rare mRNAs) could not reliably be detected against the background. The selection of interesting cDNA clones would therefore have been laborious. Instead, we decided to facilitate the screening by using probes that were enriched by subtractive-hybridization.

We chose a modification of a subtraction technique used by Wang and Brown (1991). We preferred this technique for a number of reasons. Firstly, an important advantage is the amplification of the enriched probes by PCR. Thus, in screening the libraries, the detection of clones corresponding to rare mRNAs is not limited by the amount of the probe. Secondly, for future experiments more probe can easily be regenerated. Another advantage is that multiple rounds of countercurrent subtraction generate extremely enriched probes (see Wang and Brown, 1991), thus reducing the number of false-positive clones in screenings. Furthermore, biotin/streptavidin combined with phenol extractions are used instead of hydroxylapatite chromatography (see Sargent, 1987) to remove undesired fragments. The biotin/streptavidin combination is an easy and efficient method now widely used in subtractions (see Swalla et al., 1993; Sasai et al., 1994; Rothstein et al., 1992; Lemaire et al., 1993).

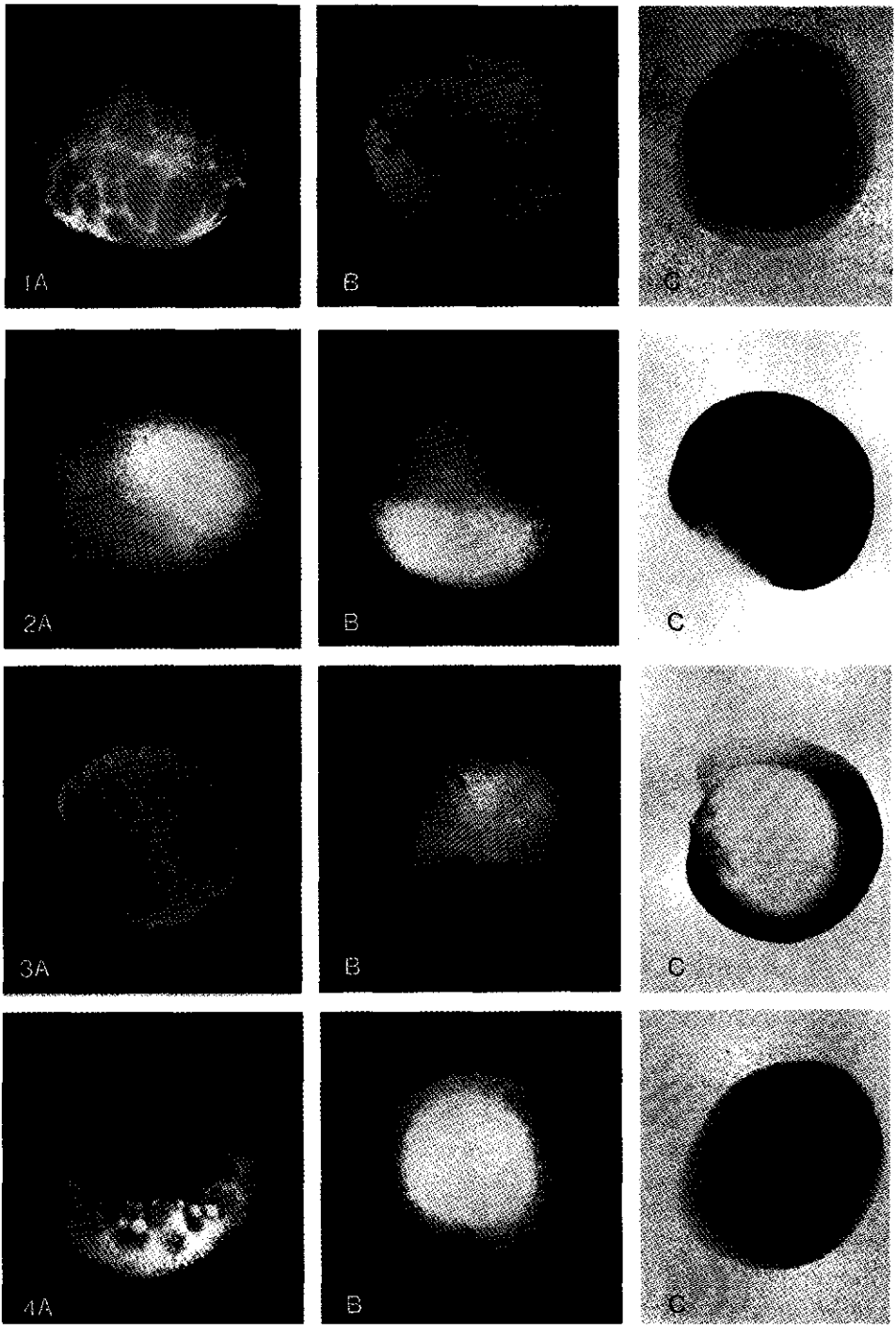
Our small-scale screenings of the carp oocyte and segmentation stage libraries with enriched probes yielded 75 positive clones. Fifteen maternal clones (65%) were specific for cleavage stages and 26 early segmentation stage-derived clones (50%) were specific for later developmental stages. Twenty-seven stage-specific clones may represent novel genes. We may have classified some clones as 'novel' which in fact represent known genes as we only partially sequenced the 5' ends and, therefore, we might in some cases have analyzed untranslated regions. These regions, as opposed to the coding region, might share less identity with related genes in evolutionary distant species.

Identified clones

The identities of clones are listed in Table 2. Sixteen clones are of mitochondrial origin (Table 2) and half of these represent the 16s rRNA transcript. The other half encode: cytochrome b, cytochrome c oxidase subunits I, II, III and NADH ubiquinone oxidoreductase subunit 2. This last group of clones displays intensive hybridization in all embryonic stages examined (Table 1). The isolation of these clones, unexpected after a subtractive hybridization, may be the result of the abundance of expression of the genes involved. Apparently, one round of subtractions was not sufficient to remove all highly abundant fragments.

The presence of ribosomal RNAs in a cDNA library is surprising but not uncommon (Sargent and Dawid, 1983; Wells et al., 1982). Recently, a 16s rRNA transcript was isolated which contained a polyA tail, polyadenylation signals and an open reading frame (Peng et al., 1992). In our case, the oligo(dT) primers used for cDNA synthesis seem to have annealed to an A-rich region in the rRNA (clone 1-15: location 2813-2831; other 16s rRNA clones: location 2884-2902 of Accession number X61010 of the EMBL database; Chang et al., 1994).

Also surprising is the stage-dependent occurrence of 16s rRNA (Table 1).


1A
B
C
2A
B
C
3A
B
C
4A
B
C

Figs. 1-4 **1A-C** Whole mount in situ hybridization of probe 1-22. Hybridization occurs uniformly in the cleavage stage embryo (**A**), but at 40-50% epiboly the staining is confined to a ring around the margin as well as a region of cells presumably representing the embryonic shield (**B**). No hybridization is found in the segmentation stage (**C**). **2A-C** Whole mount ISH of probe 3-36, representing a gene with stage-specific, but not localized expression. 3-36 mRNA is not present in the cleavage stage (**A**). Hybridization is ubiquitous at 50% epiboly (**B**) and in the segmentation stage embryo (**C**). **3A-C** Whole mount ISH of probe 3-28. Cleavage stages contain no 3-28 mRNA (**A**). At approximately 50% epiboly, hybridization is most intense in the germ ring and embryonic shield (**B**; animal pole view). In the segmentation stage embryo (**C**), the hybridization is localized in the posterior part of the embryo. **4A-C** Whole mount ISH of probe 3-55. The 3-55 mRNA is present in all blastomeres in the cleavage stage (**A**), and in the involuted cells during epiboly (**B**; animal pole view at approximately 50-60%). In the segmentation stage embryo (**C**), a small region posterior to the eyes is intensely stained.

We are not aware of previous reports dealing with the disappearance of mitochondrial rRNA transcripts during embryonic development and, so far, we have not been able to explain this phenomenon. The stage-dependency in ISH does, however, account for the isolation of the clones after subtractive hybridizations.

In addition, we identified five keratin clones (Table 2). The expression of keratin was restricted to post-cleavage stages (Table 1) and transcripts were localized on the outside of the embryo, presumably in the enveloping layer. These results agree with the expression pattern described for a type II cytokeratin identified in *Xenopus laevis,* XenCK55(5/6) (Fouquet et al., 1988).

Clones for further study

Many genes with localized expression patterns have been reported to be important for determination and/or differentiation processes in early development (see Dawid, 1994; Kessler and Melton, 1994; Beddington and Smith, 1993). In light of this, we selected the clones 1-22, 3-28 and 3-55 for future experiments dealing with the roles of these genes during embryonic development.

The 1-22 mRNA is present before segmentation stages. Its expression in cleavage stages and the subsequent localization of the transcripts in the germ ring and embryonic shield is also described for *snail1* in zebrafish (Thisse et al., 1993). Expression of the 1-22 gene after 50% epiboly differs from *snail1*, as 1-22 mRNA disappears before segmentation stages. The expression pattern of 1-22 suggests it plays a role during the gastrulation process, perhaps in regulating the determination or differentiation of the hypoblast. To our knowledge, an expression pattern like that of clone 1-22 has not been described before.

The 3-28 gene is expressed in the germ ring and embryonic shield at 50% epiboly and posteriorly in segmentation stage embryos. The posterior localization in segmentation stages resembles the expression of *caudal*, a gene involved in gastrulation and patterning along the anteroposterior (a-p) axis of the embryo (Joly et al., 1992; Stroband et al., 1995). By analogy to *caudal*, 3-28 might also be involved in pattern formation of the a-p axis of the embryo. The gene has been almost completely sequenced and is being analyzed. The clone might represent a novel gene as we have found no homology as yet to caudal or any other known gene.

3-55 mRNA is mainly present in involuted cells at 50-60% epiboly and during segmentation stages in a region of the brain, behind the eyes. Sequence analysis and the ISH pattern of expression indicate that clone 3-55 is identical to zebrafish *Otx1*, an orthodenticle-related homeobox-containing gene. The *Otx* genes are the earliest neurally expressed genes identified in the zebrafish (Li et al., 1994) and they are thought to play a role in specification of forebrain and midbrain and the subsequent subdivision of the diencephalon and the midbrain (Li et al., 1994; Mori et al., 1994).

Clones 3-28 and 3-55 are interesting as markers for studies of positional specification of the a-p axis, with 3-28 as a posterior and 3-55 as an anterior marker. In particular, 3-55 (*Otx1*) could be important for studies of anterior head and CNS development.

Materials and methods

Embryos and RNA isolation

Carp sperm and oocytes were obtained as described in Stroband et al. (1995). Fifty to 100 g oocytes were mixed with 1 to 5 ml sperm and fertilized in urea/NaCl solution (0.3% urea / 0.4% NaCl in Cu^{++}-free water). Fertilized eggs were incubated in this solution for 30 minutes to prevent their agglutination. After a thirty-seconds incubation in tannic-acid solution (0.35 g/l), the embryos were cultured at 25°C in Cu^{++}-free water in a Zugerglas. Embryos and oocytes were randomly selected, frozen in liquid nitrogen and stored at -80°C for RNA extractions. Embryos were only used if the morphology of more than 90% of the control embryos appeared normal after 24 hours post fertilization.

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). After homogenization of the embryos in solution D, chorions were removed by centrifugation (5-10 minutes, approximately 800 g, 4°C), as they disturb the subsequent phenol-chloroform extraction. Poly(A)$^+$ RNA was selected with a PolyATtract mRNA isolation system (Promega, Madison, WIS.; for construction of cDNA libraries) or with an oligotex-dT mRNA kit (Qiagen, Düsseldorf, Germany; for subtractive hybridization).

Construction of cDNA libraries

The oocyte cDNA library (1.2 x 10^6 pfu) and early-segmentation stage (first pairs of somites) cDNA library (1.3 x 10^6 pfu) were created with the ZAP-cDNA synthesis kit and the Gigapack II Gold Packaging Extract (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions.

Strategy

For subtraction of the early-segmentation stage from the oocyte stage, the oocyte stage served as tracer and the segmentation stage as driver. For subtraction of the oocyte stage from the early-segmentation stage, driver and tracer were exchanged. The subtraction technique applied in this paper follows the gene-expression-screen technique as described by Wang and Brown (1991), unless indicated otherwise.

cDNA for PCR amplification

Double-stranded cDNA was synthesized from 10 μg poly(A)$^+$ RNA using the RiboClone cDNA synthesis system (Promega, oligo(dT)$_{15}$ primer). cDNA fragments suitable for PCR amplification were prepared as described by Wang and Brown (1991). Oocyte and segmentation stages were treated identically during all procedures. The fragments were amplified by PCR (94°C, 1 minute; 50°C, 1 minute; 72°C, 2 minutes; 30 cycles) to produce sufficient cDNA for subtractive hybridizations.

Subtractive hybridization

The driver cDNA was photobiotinylated twice (30 minutes each time) with Photoprobe biotin (Vector Laboratories inc., Burlingame, Calif.). For irradiation we used a 160 Watt ML bulb (Philips Nederland BV, Eindhoven, The Netherlands). For subtractive hybridizations we used half the amounts described by Wang and Brown (1991). The enriched tracer cDNA, obtained after one "long" and one "short" hybridization was precipitated and dissolved in 30 μl TE buffer. 2 μl were used in a 40 μl PCR reaction (200 μM each dATP/dCTP/dGTP, 150 μM dTTP, 50 μM DIG-11-dUTP (Boehringer Mannheim BV (BM), Almere, The Netherlands) to generate the enriched probe for differential screening.

Differential screening

The differential screening was carried out for both libraries, as follows. Duplicate plaque lifts were made of approximately 600 pfu. The filters were prehybridized for 2 hours at 42°C in 5 x SSC / 0.1% w/v lauroylsarkosine / 0.02% w/v SDS / 2% blocking stock (BM) / 50% deionized formamide / 20 μg/ml *E. coli* DNA (Pharmacia, The Netherlands) and hybridized for 80 hours under the same conditions. One filter was hybridized to the oocyte-enriched probe and the other to the segmentation stage-enriched probe. Washings: 2 times 5 minutes with 2 x SSC / 0.1% SDS at room temperature and 2 times

20 minutes with 0.1 x SSC / 0.1% SDS at 42°C. The DIG detection followed the manufacturer's protocol (BM).

Sequence analysis

Sequencing was performed as described in Stroband et al. (1995). The similarity searches were carried out with the FASTA programs (Pearson and Lipman, 1988) in the GenEMBL databases. Clones 3-14, 3-51 and 3-56, isolated from the segmentation stage library, could not be cultured and were omitted from this study.

RNA probes and In Situ Hybridization

Six μg plasmid DNA was digested with EcoRI, phenol-chloroform and chloroform extracted, subsequently precipitated with ethanol and used as template for the transcription reaction. DIG-labeled antisense RNA probes were synthesized with T7 polymerase using the DIG RNA-labeling kit (BM). Probes were diluted 2000 - 4000 times for in situ hybridization (ISH). The hybridization of each probe was studied for 5 developmental stages (cleavage stage, late blastula stage, approximately 50% epiboly, approximately one and 8 pairs of somites). ISH was performed as described by Stroband et al. (1995), with a modified hybridization buffer (50% formamide, 2 x SSC, 0 - 2% blocking solution, 0.1 - 2% Tween-20, 5 mg/ml yeast torula RNA, 50 μg/ml heparin). For photography, segmentation stage embryos were dehydrated in methanol, isopropanol and then mounted in tetralin (Aldrich Chemie, Brussels, Belgium).

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Chapter 5

Blastomeres and cells with mesendodermal fates of carp embryos express cth1, a member of the TIS11 family of primary response genes

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submitted

Abstract

The carp *cth1 gene*, related to the mammalian *TIS11* family of primary response genes, encodes a novel fish protein with two putative CCCH zinc fingers. This report describes the RNA expression of this gene during cleavage, blastula and gastrula stages of carp embryos.

Cth1 mRNA is present in all cleavage stage blastomeres as a maternal message. After the late blastula stage, the maternal expression decreases, revealing a spot of higher expression at the margin of the blastoderm of the dome stage embryo. Further decrease of the maternal message reveals a ring of *cth1* expressing cells at the blastoderm margin from the stage of 40% epiboly onwards. By α-amanitin treatment we established that this local *cth1* expression is of zygotic origin.

At the onset of gastrulation the cells of the *cth1* ring involute, starting with those in the shield region, and at approximately 60% epiboly the ring is fully involuted and occupies the hypoblast layer. All *cth1* transcripts have disappeared at completion of epiboly. We discuss a possible role for the putative cth1 protein in preserving cellular potential in cells at the margin of the blastoderm, and in cells of cleavage stages.

Introduction

A great deal of attention is being paid to the molecular mechanisms that underlie the induction and differentiation of germ layers in teleost fish, especially zebrafish (*Danio rerio*). Gastrulation forms the epiblast and hypoblast, two layers of cells that give rise to respectively the ectoderm, and the mesoderm and endoderm (Kimmel et al., 1995). Initially, the prediction of a cell's future could only be made just prior to gastrulation (Kimmel et al., 1990) as cell mixing was thought to randomly rearrange the positions of cells in the blastula (Warga and Kimmel, 1990). Recently it became clear that in spite of this cell mixing, mesoderm and endoderm arise from only a subset of cells in the blastula (Kimmel et al., 1990; Helde et al., 1994), namely the cells at the margin of the blastoderm (hereafter referred to as 'marginal cells'). These marginal cells undergo but limited mixing and therefore keep their position near the margin of the blastoderm (Wilson et al., 1993; Helde et al., 1994) and then occupy the germ ring. Cells in the germ ring involute or ingress during gastrulation (reviewed by Solnica-Krezel et al., 1995) and become the hypoblast layer. Though inducing signals are provided to the blastomeres before the onset of epiboly and gastrulation (Mizuno et al., 1996; Bozhkova et al., 1994; te Kronnie et al.), they are not determined until midgastrulation (Ho, 1992; Ho and Kimmel, 1993). How this signaling leads to specification is still unresolved. Analysis of gene expression patterns in the margin of the

blastoderm, for example of circumferentially expressed genes like *no tail* (Schulte-Merker et al., 1992), *lim1* (Toyama et al., 1995) and *snail* (Hammerschmidt and Nüsslein-Volhard, 1993; Thisse et al., 1993, 1995), provided some insight in the process. In this light we describe the expression of a novel fish gene that may be involved in the formation of the mesendoderm (mesoderm and endoderm), in carp (*Cyprinus carpio*) embryos. Carp is a teleost fish with identical development to zebrafish. The gene was previously isolated by subtractive hybridization (Stevens et al., 1996b) and we now identify it as belonging to the *TIS11* family of primary response genes. *TIS11* family members, also reported as *TTP, Nup475, TIS11, cMG1, CTH, ERF,* are expressed in a wide variety of tissues and species (see references in discussion) and are induced after treatment with tumor promoters and growth factors, independent of protein synthesis (Herschman, 1991; Kaneda et al., 1992; Bustin et al., 1994). As the encoded proteins contain highly conserved putative zinc fingers, they are generally thought to regulate gene expression.

Here we report that the carp gene is expressed in all blastomeres at cleavage and blastula stages and is subsequently restricted to cells at the margin of the blastoderm and the germ ring. During gastrulation, the cells with transcripts involute and contribute to the hypoblast. We discuss the possible mode of action and propose a developmental role for this gene.

Results

Sequence analysis

We previously isolated the carp *cth1* clone (Fig. 1) by subtractive hybridization of oocyte cDNA with that of early segmentation stage embryos (Stevens et al., 1996b). The clone has a length of 1438 nucleotides (nt) with an open reading frame extending from position 133 to the stopcodon at 810. It encodes a protein of 226 amino acids (aa). The 3′ untranslated region (UTR) of 626 nt is complete, as indicated by the presence of a polyadenylation signal (AATAAA). Four possible signal sequences of unstable mRNAs (ATTTA) are recognized in the 3′UTR (Shaw and Kamen, 1986).

By Fasta and Blast searches we identified a 65 aa motif within the deduced carp protein, which is the characteristic feature of the group of mammalian and fruitfly *TIS11*, and yeast *CTH* genes. Carp cth1 is most closely related to the mouse and human TIS11d proteins (69% identity in motif). Members of the TIS11/CTH class share an unusual putative zinc finger motif (CCCH) in a consensus sequence **C**-x6-G-x-**C**-x-Y-x3-**C**-x-F-x-**H**, which is repeated and preceded by the conserved YKTEL sequence (Fig. 2). In addition, the spacing between the two repeats is conserved (Fig. 2). The carp motif owns all these features and therefore we identified

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   1  GCGTCTTTCATCTGGAATAGAGCAGCGGAGTCAAGTGTTTGTCCGGAGTGCAGTCGGGTT
  61  TAGCGTCACTTTTTAAACGACAACCAACCAAGTTTTGTTAAGCTGCTGTTGAGTTTTGTA
 121  AAGCTGTCTAAAATGTTTGAGACTAGTACAGATAACCTGTTTCTGTTCCCCACTGAAGGC
                  M  F  E  T  S  T  D  N  L  F  L  F  P  T  E  G
 181  CTGAATGAGGCTTTCTTCCCTGAAGAGGGTTTAGCCAGTGGGAGCTTGTCTCTTGCCAAG
      L  N  E  A  F  F  P  E  E  G  L  A  S  G  S  L  S  L  A  K
 241  GCCTTGCTTCCCCTGGTTGAGTCCCCATCACCCCCGATGACGCCCTGGCTCTGCTCCACC
      A  L  L  P  L  V  E  S  P  S  P  P  M  T  P  W  L  C  S  T
 301  CGCTATAAGACAGAACTGTGCAGCCGATATGCTGAGACAGGTACCTGCAAGTATGCCGAA
      R  Y  K  T  E  L  C  S  R  Y  A  E  T  G  T  C  K  Y  A  E
 361  CGCTGCCAGTTCGCCCATGGACTCCATGATCTCCACGTACCCTCCCGTCATCCCAAGTAC
      R  C  Q  F  A  H  G  L  H  D  L  H  V  P  S  R  H  P  K  Y
 421  AAAACCGAGCTGTGCCGTACCTACCACACTGCTGGCTACTGTGTCTATGGCACACGGTGT
      K  T  E  L  C  R  T  Y  H  T  A  G  Y  C  V  Y  G  T  R  C
 481  CTCTTTGTGCACAACCTTAAAGAGCACAGGCCTGTCCGTCAACGGTGCAGGAACGTGCCT
      L  F  V  H  N  L  K  E  H  R  P  V  R  Q  R  C  R  N  V  P
 541  TGCCGTACCTTTCGTGCATTTGGGGTTTGCCCTTTGGTACCAGATGCCACTTCCTGCATG
      C  R  T  F  R  A  F  G  V  C  P  L  V  P  D  A  T  S  C  M
 601  TGGAGGGTGGTTCAGAATCAGATGGTGGAGAGGAAGAGCAAACCTGTCAACCTATGTCAC
      W  R  V  V  Q  N  Q  M  V  E  R  K  S  K  P  V  N  L  C  H
 661  AGTCCCAAGAGTGGAAGCCTCGAGGTGCCCTCTGTCGCACCTTCAGCGCTTTTGGTTTCT
      S  P  K  S  G  S  L  E  V  P  S  V  A  P  S  A  L  L  V  S
 721  GTCTCTATGGCACCCGTTGTCGATTCCAACATGGGCTTCCCAACTCGATCAAAGGTGTCA
      V  S  M  A  P  V  V  D  S  N  M  G  F  P  T  R  S  K  V  S
 781  ACTCAACCCACACATCCTGGCCTCATCAGATGACCAATAGGGGATCTCTTTCACCTGTGT
      T  Q  P  T  H  P  G  L  I  R
 841  CAGATGCGTGCTCGTCACAATCTCCACCGTCCTCGTCCCCTCCGTCTGCGTTGGCTTCGC
 901  GGTGTACCCTGAGGGTTCTGGTCCAGTCACACCACCATCGGTAGAAGCAGTAGCCAACAA
 961  CGCTTTCACCTTCAGCAGCCAACATCTGAATGACCTTCTGCTCCCCCTGGCCCTTCGGCT
1021  CCAGCAGCTGGAGAATGTTACCAATGCTGGTCCTCAAGATGCTGTGGATAAGCCACTGTT
1081  GTTAAGTCTGTGGCAAGATGATCCAAGAAGCTAACTGCTGTGAGCTTTAACTGTGACTGT
1141  ATTGTGCCAAATTTATATTAACAGTTTTTATTTAGTTGTGTACAGTGTCTCTTTGCAACC
1201  TATTTTTAACTGCCTTTTTTAATTCAAGTCTGTCTTGTACTTCTGAGGCCACTTATTTTT
1261  GACTGACTGAATGAAGCCCCTTAAGTGTAAATTGAGACCACTTTACTGGTGTATTTATGA
1321  AGGATTTGACTATGTATGTTTCTAAATTACGGATTTTAAAACTTTAATGACTTAAATTAT
1381  TTTAAAAACATCAGCATCCTTTGCTCTCAATAAAACCTCCTAAAAAAAAAAAAAAAAA
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Fig. 1 Nucleotide sequence and predicted protein product of carp *cth1*. The instability conferring motifs are single underlined and the polyadenylation signal is double underlined. The putative zinc finger is printed in bold.

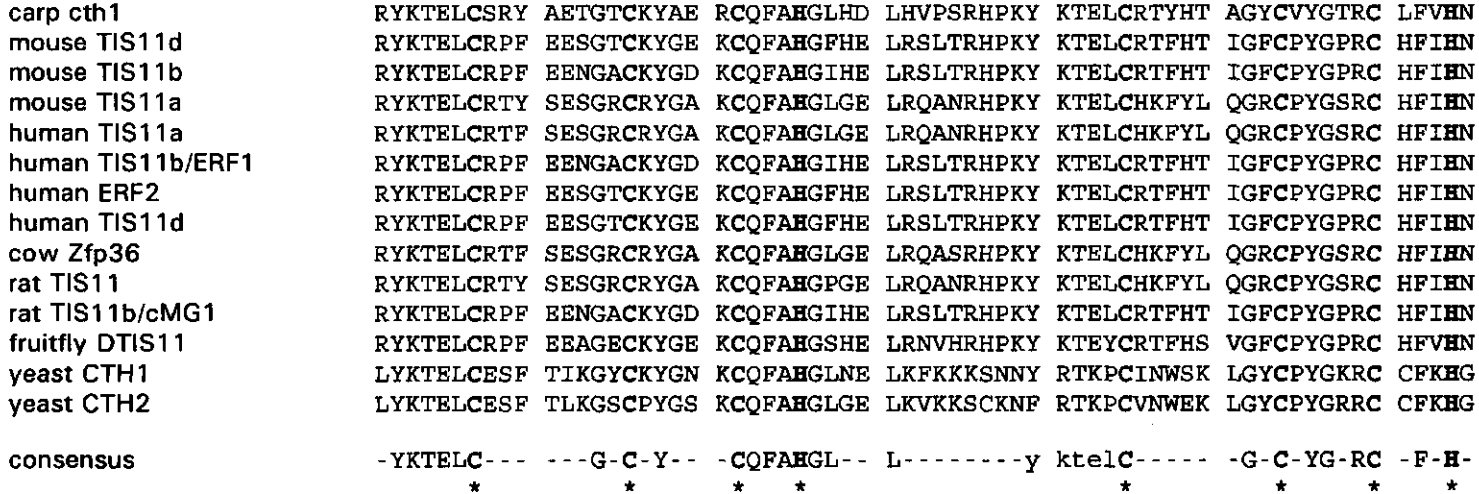

Fig. 2 Comparison of the zinc finger regions of carp cth1 and other members of the TIS11 family. The two zinc binding motifs (CCCH) are spaced by 18 amino acids. Zinc binding residues are indicated by asterisks. The CCCH motifs are preceded by a conserved YKTEL sequence, which is less conserved in the second motif. The TIS11 sequences are available under the following accession numbers: carp cth1 (Y10163); mouse TIS11d (P23949); mouse TIS11b (B39590); mouse TIS11a (P22893); human TIS11a (P26651); human TIS11b/ERF1 (Q07352); human ERF2 (S49147); human TIS11d (U07802); cow Zfp36 (L42319); rat TIS11 (JC1255); rat TIS11b/cMG1 (P17431); fruitfly DTIS11 (U13397); yeast CTH1 (P47976); yeast CTH2 (P47977). The mouse and human TIS11a are also known as TIS11, Zfp36, Nup475, or TTP.

the carp gene as the first fish *TIS11*-like gene. We followed the nomenclature suggested for the yeast genes (Thompson et al., 1996) and named it *cth1* (cysteine-three-histidine).

Northern blot analysis

Carp *cth1* transcripts were present from the oocyte stage until the end of gastrulation (see below). To obtain further information concerning transcript sizes and the decrease of the expression level we analyzed the *cth1* signal in successive developmental stages on a northern blot (Fig. 3). *Cth1* RNA was abundantly present during the oocyte and cleavage stages. Around 5 hours (oblong stage), the expression decreased to a lower level and persisted throughout gastrulation stages. At the start of somitic segmentation, the expression had disappeared. The *cth1* mRNA had not reappeared at 24 hours of development (Fig. 3). In addition, the northern blot demonstrated a one-sized transcript for all developmental stages analyzed.

Carp cth1 RNA expression in situ

Carp embryonic development (Neudecker, 1976; Stroband et al., 1992, 1995; Stevens et al., 1996a) is similar to that of the closely related zebrafish, *Danio rerio* (Kimmel et al., 1995). Through cleavage and blastula stages, maternal *cth1* transcripts were present in all blastomeres of the embryo (Fig. 4A), without any apparent localized pattern. The first unevenness in the expression pattern arose just before doming when the intensity of expression in the blastoderm had decreased (Fig. 3). This reduction of overall expression revealed a spot at the margin of the blastoderm (Figs. 4B, 4F). Concomitant with the optical reduction of the maternal signal in the blastoderm, caused by its thinning during doming and epiboly, a ring of *cth1* expressing cells, including the former spot, was distinguished at the margin (Figs. 4C, 4G). The expression in these cells was observed during completion of epiboly, while the signal in other blastoderm cells gradually diminished and disappeared before the end of gastrulation (Figs. 4A-E).

Gastrulation begins at 50% epiboly with the involution of cells at the margin of the blastoderm (Kimmel et al., 1995). Cells that remain in the outer layer form the future epiblast, those in the inner layer form the hypoblast. At the onset of gastrulation, *cth1* cells in the ring at the margin involuted, starting in the spot on the dorsal side (Fig. 4D). At approximately 60 - 70% epiboly, the *cth1* expressing ring of cells had completely involuted and occupied the early hypoblast layer (Figs. 4E, 4H). The *cth1* signal in the hypoblast decreased as gastrulation proceeded and was undetectable at the end of epiboly.

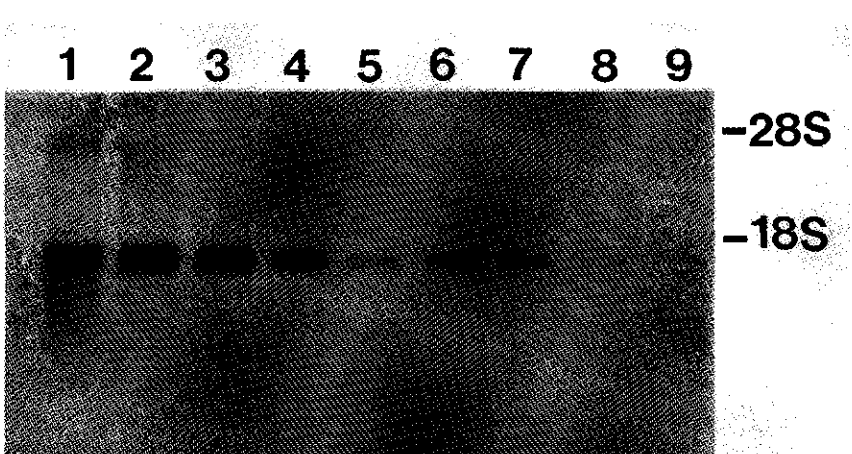

Fig. 3 Northern analysis of *cth1* RNA. Lanes 1-9 contain 2 μg total carp RNA from: 1) 0 hours (oocyte), 2) 2 hours (32/64 cells), 3) 4 hours (high), 4) 5 hours (oblong), 5) 5½ hours (early dome), 6) 6 hours (30% epiboly), 7) 6½ hours (40-50% epiboly), 8) 7 hours (± 60% epiboly), 9) 24 hours (late segmentation). Lanes 1 and 2 are respectively slightly underloaded and overloaded. The positions of the ribosomal RNAs are shown.

A maternal origin for cth1 transcripts in marginal cells?

The localized pattern of *cth1* transcripts at the margin of the blastoderm during epiboly and gastrulation raised the question whether these transcripts were of maternal origin, or resulted from de novo transcription of the embryonic genome. Unstable mRNAs, containing ATTTA instability motifs as found in the carp mRNA, can be stabilized by mRNA binding proteins (Jacobs et al., 1996), and thus the circumferential *cth1* expression could be the result of selective stabilization of maternal transcripts in marginal cells, while those in other cells are degraded. Alternatively, this pattern could be zygotic as it arised after genome activation occurs (around the 10th cleavage in carp: Stroband et al., 1992). To resolve this question, we blocked the embryonic transcription with α-amanitin, a drug that inhibits RNA polymerase II. If the *cth1* pattern is still observed after administration of this drug, the circumferential expression must be of maternal origin.

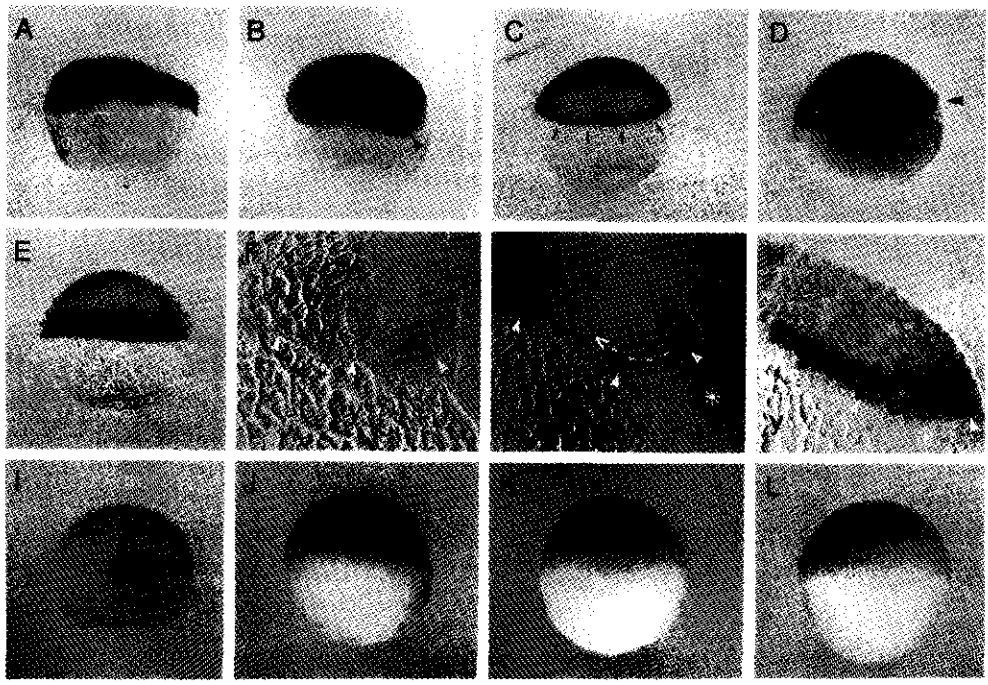

Fig. 4 *Cth1* RNA distribution in carp embryos. **A** Cleavage stage (16 cells) embryo has maternal *cth1* RNA in all blastomeres. **B** Maternal *cth1* expression has become less distinct. A spot of higher expression is visible at the margin of the blastoderm (arrowhead). **C** 40% epiboly; a ring of *cth1* expressing cells is located at the margin of the blastoderm (arrows). Maternal RNA is still ubiquitously present at low level in the rest of the blastoderm. **D** 50% epiboly; the *cth1*-ring involutes during gastrulation, starting in the shield region (arrowhead). Note that the half of the circle which faces away from the reader is visible through the yolk (upper line), whereas in **E** only the front half of this circle can be seen. The irregular shape of the *cth1*-circle is caused by unevenness of the blastoderm margin, which often occurs in carp epiboly. **E** The *cth1*-circle is fully involuted at 60% epiboly and occupies the hypoblast layer. The blastoderm outline is marked with a dotted line. Dorsal is to the right. **F-H** Nomarski images of sectioned embryos. Asterisk indicates the YSL, with arrowheads pointing at the border between blastoderm and YSL. y = yolk, b = blastoderm. **F** and **G** are understained, therefore the overall low-level expression in the blastoderm is not visible. **F** Section of **B** (dome stage), through the spot of

(Fig.4 continued) - *cth1* expressing cells at the blastoderm margin. **G** Section through **C** (*cth1*-ring at 40% epiboly. Note that the blastoderm layer has thinned during epiboly (compare **G** to **F**). The *cth1* expressing cells of the ring are localized in the margin of the blastoderm and will involute during gastrulation (arrow indicates direction of movement). **H** Section through **E** (60% epiboly) showing that *cth1* expressing cells have involuted into the hypoblast. There still is overall low-level expression in the blastoderm. **I** Carp embryos (60% epiboly) show a normal pattern for zebrafish *goosecoid* (arrowhead). **J-L** *Cth1* expression in α-amanitin-treated carp embryos fixed when normal embryos had reached dome (**J**), 30% (**K**) and 60% epiboly (**L**). *Cth1* RNA is stained brown instead of blue. The overall expression in the blastoderm is of maternal origin and disappears gradually, as in normal embryos (**B**, **C**, **E**). The localized expression pattern does not arise and is therefore most likely of zygotic origin.

α-amanitin treatment

Alpha-amanitin was injected into early cleavage stage embryos (n=167). These embryos appeared normal in cleavage stages, but their development became aberrant when epiboly started in untreated embryos. Though not completely arrested in their development since cell divisions were still observed, α-amanitin embryos did not undergo epiboly or gastrulation. They maintained a late-blastula like shape until degenerating after 10 hours of development.

First, we confirmed the inhibition by α-amanitin of embryonic genome activation by the absence of transcripts from a zygotically expressed gene, zebrafish *goosecoid*. *Goosecoid* was not detected in these embryos, contrary to its normal expression in control carp embryos (Fig. 4I), demonstrating that zygotic gene expression was indeed blocked. *Cth1* expression in α-amanitin treated embryos was examined when untreated embryos had reached dome (Fig. 4J), 30% epiboly (Fig. 4K) and approximately 60% epiboly stages (Fig. 4L). Only the ubiquitous signal that resulted from the maternal expression in cleavage stages was detected. The localized expression in the spot and in a ring around the margin as seen in control embryos lacked in perturbed embryos, indicating a zygotic origin of this pattern in normal development.

Discussion

Carp cth1 belongs to the TIS11 gene family

The *TIS11* family of primary response genes is characterized by a repeated sequence motif that is supposed to encode a novel type of zinc finger (CCCH). Zinc-binding by the putative finger was recently proven (Worthington et al., 1996), and the secondary structure of the zinc binding domain described. Another shared motif among the *TIS11* genes is

the YKTEL pentapeptide. The putative carp protein has the characteristic features of TIS11 proteins and therefore we identified it as belonging to the *TIS11* gene family.

Members of this family were found in mouse and rat (Herschman, 1991), humans (Nie et al., 1995), *Drosophila* (Ma et al., 1994), cow (Lai et al., 1995) and yeast (Thompson et al., 1996). Except for truly homologous TIS11 proteins, such as human TIS11d/mouse TIS11d (Ino et al., 1995), human ERF-1/mouse TIS11b/rat cMG1 (Barnard et al., 1993), the similarity outside the zinc finger domains between the different TIS11 proteins is low. Though the zinc finger domain of carp cth1 is quite similar to that of mouse and human TIS11d (70%), an overall aa comparison reveals only little similarity (35%), suggesting that TIS11d proteins are not true equivalents of the carp protein. It is likely that many more *TIS11* genes are still to be discovered and a true homolog of carp *cth1* may be found among them.

TIS11 expression during development

Little is known about the developmental expression of *TIS11* genes in different species. In carp embryos, *cth1* transcripts were found until the end of gastrulation. Maternally supplied transcripts were ubiquitously spread over the blastomeres during the cleavage period. After the late blastula stage, these transcripts were degraded and had disappeared before the end of gastrulation. *Drosophila TIS11* (*DTIS11*) is also expressed during early embryonic development (Ma et al., 1994). *DTIS11* is abundantly present all over the embryo during the rapid nuclear divisions, comparable to the expression of carp *cth1* during cleavages, and starts to decline when cellularization starts. A localized pattern of carp *cth1* expression became distinct during epiboly when the maternal expression disappeared. First seen as a spot at the margin of the blastoderm at the dome stage, *cth1* expression completely encircled the blastoderm margin during epiboly and involuted when gastrulation started. As in carp, fruitfly *DTIS11* mRNA remains present after the cleavage period (Ma et al., 1994) but it is not clear if these transcripts are localized, like the carp transcripts are.

If classes of *TIS11* genes exist, they may have distinct developmental expression patterns and gene-specific modes of transcriptional regulation. Differential regulation has already been demonstrated *in vitro* for *TIS11* and *TIS11B* (Varnum et al., 1991). It is interesting to know to what extent the expression patterns of those *TIS11* classes are comparable between species. Conservation of developmental patterns is suggestive of conserved roles during development.

Maternal versus zygotic transcription

The question whether the localized *cth1* pattern during epiboly arised as

a result of zygotic transcription or consisted of remnant maternal RNA is an intriguing one. On the one hand, localized zygotic expression preceded by maternal expression is commonly seen in embryos (for example *goosecoid*: Stachel et al., 1993; *snail1*: Thisse et al., 1993) and could explain the pattern of *cth1* in post-cleavage carp embryos.

On the other hand, arguments exist that favor the maternal origin of this pattern. Firstly, maternal *cth1* transcripts were still abundantly present when the localized pattern became distinct, in contrast to the maternal *goosecoid* RNA that disappears before zygotic transcripts appear (Stachel et al., 1993). Secondly, zygotic transcription could be expected to increase the abundance of *cth1* mRNA, but the northern blot showed a decline of the level of expression instead. Moreover, carp embryos showed no other size of *cth1* transcript than the maternal one, in contrast to fruitflies that have an embryonic transcript of different size (Ma et al., 1994). These arguments led us to consider a maternal origin for the localized mRNA. The model that we proposed was one of selective stabilization of maternal transcripts, and would provide an interesting mechanism for localization of expression. *TIS11* RNAs rapidly degrade and this instability is attributed to ATTTA sequences (Shaw and Kamen, 1986) which are also present in the 3'UTR of carp *cth1*. A half-life less than 30 minutes is reported for *TTP* mRNA (Lai et al., 1990). Since the *cth1* levels remained high until the age of 5 hours, we expected the maternal *cth1* RNA to be stabilized. After the cleavage stages, a stabilization of the RNA might occur only in the margin of the blastoderm, giving rise to a circle of expression while transcripts in other parts of the embryo are degraded.

However, no localized expression was found after treatment with α-amanitin thereby making the existence of a mechanism of selective stabilization improbable. We conclude that the localized pattern most likely consists of zygotic *cth1* transcripts.

Induction of zygotic expression

Why is zygotic *cth1* transcription restricted to cells in the margin of the blastoderm? An explanation may be found in recent reviews (Grunwald, 1996; Stroband et al., 1996) that suggest that maternally supplied substances, emitted from the yolk cell, are responsible for induction processes in marginal cells. The mammalian *TIS11* genes are rapidly induced by tumor promoters and growth factors (see references in introduction). The zygotic *Drosophila TIS11* RNA is also inducible (Ma et al., 1994). By analogy it is likely that zygotic *cth1* expression in carp reflects a primary response to an inducing factor. The above model suggests that this substance, for example a growth factor (Stroband et al., 1996), originates from the yolk cell. Cells in close contact with the YSL are thought to receive this inducing signal and this could explain why *cth1* transcription is only activated in a ring of cells around the margin of

the blastoderm.

Possible function of the cth1 protein

The RNA expression pattern suggests that *cth1* translation could happen twice; first from maternal mRNA in blastomeres during the cleavage and blastula periods, and then from zygotic mRNA in marginal cells during epiboly. However, many factors are known to regulate mRNA translation in embryos (Wolffe and Meric, 1996; MacDonald and Smibert, 1996; Wormington, 1993). Therefore, *cth1* translation does not necessarily occur in all parts of the RNA expression pattern.

It is assumed that the mechanism by which TIS11 proteins act involves the conceptual zinc finger. Zinc finger proteins are generally considered regulators of transcription (Pabo and Sauer, 1992). Recently, evidence for RNA binding by a *Xenopus* zinc finger protein (Andreazzoli et al., 1993) suggested a second mode of action. Cytoplasmic Xfin protein is thought to bind target mRNAs and thereby regulate their translation. Phosphorylation of Xfin should lead to tight binding whereas dephosphorylation should release the target mRNA (Vignali et al., 1994). Although, for the present, evidence for RNA-binding by TIS11/CTH proteins is lacking, three facts suggest a similar mode of action. Firstly, the existence of only transcriptional regulation would exclude a role for the maternal protein, as the fish genome is not yet transcribed during early cleavages. Thus, a role in translation is more plausible. Secondly, the mouse TTP protein is rapidly translocated from the nucleus to the cytoplasm in response to mitogens (Taylor et al., 1996b), suggestive of a cytoplasmic function and thus favouring translational over transcriptional regulation. Thirdly, the TTP protein is phosphorylated and this may regulate its function (Taylor et al., 1995), as suggested for Xfin.

As to the nature of gene regulation, inhibiting roles have been attributed to TIS11 proteins. For example, overexpression of CTH in yeast leads to impairment of growth and this is probably mediated through the zinc finger domain (Thompson et al., 1996; Ma and Herschman, 1995). In mice, TTP is thought to have a role in negative regulation of TNFα (tumor necrosis factor) synthesis (Taylor et al., 1996a). We propose that carp cth1 regulates gene expression by inhibition of translation of distinct target mRNAs. The impairment of the translation of mRNAs, coding for proteins that respond to inducing signals, could be a mechanism to time or prevent the selection of certain differentiation pathways. Such a function could be used in development to prevent premature restriction of cellular potential. In support of this hypothesis is the recent finding that a protein with TIS11-like zinc fingers (PIE-1) in *C. elegans* has a comparable mode of action and preserves pluripotency in germ cells (Mello et al., 1996).

For both the maternal and the zygotic protein, a role in maintaining cellular potential is conceivable. During cleavages, blastomeres remain

pluripotent (Helde et al., 1994; Kimmel and Warga, 1987). Maternal cth1 protein could be involved in this. The zygotic cth1 may be implicated in the commitment of the mesendodermal fate. Expressing *cth1* RNA, the marginal cells involute during gastrulation and form the hypoblast layer, which gives rise to mesoderm and endoderm (Warga and Kimmel, 1990; Kimmel et al., 1990). Though marginal cells receive mesoderm-inducing signals already early (Bozhkova et al., 1994; Mizuno et al., 1996), a hypoblast-fate is not determined until midgastrulation (Ho and Kimmel, 1993) and cth1 protein could perform a delaying role in this determination. Alternatively, the protein could bring about the choice for a particular fate. How cells acquire the endodermal fate is still mysterious. Perhaps it is the default choice of hypoblast cells that do not possess appropriate proteins to respond to mesoderm-inducing signals. It is tempting to speculate that by preventing the translation of such proteins in a subset of the marginal blastomeres, cth1 could favour the selection of the endodermal fate.

To conclude

The binding of cth1 to distinct mRNAs and its regulation of their translation remain hypothetical for the time being. Experiments dealing with the DNA/RNA binding capacity of the supposed zinc finger and the identification of possible target mRNAs will be needed to resolve this issue. A comparison between the expression patterns of *cth1* and those of other genes expressed in the blastoderm margin, such as *no tail* and *snail*, can contribute to a better understanding of the developmental role of *cth1*. This role could be an intriguing one in maintaining cellular potential, especially in mesendoderm specification. Important information may be gained from the study of the distribution of *cth1* mRNA in mutant zebrafish embryos.

Materials and methods

Embryos and α-amanitin injections

Carp oocytes were in vitro fertilized. Sperm and eggs were obtained by squeezing, after stimulation with pituitary suspension as described by Stroband et al. (1992).

Alpha-amanitin (Sigma-Aldrich Chemie BV, the Netherlands), was injected during the 4 to 32 cell stages, in a final concentration in the embryo of approximately 2 - 7 μg/ml (volume embryo was estimated 0.7 μl). For use in in situ hybridization (ISH), embryos were fixed when controls (injected with equal volume 0.9% NaCl) had reached dome, 30% epiboly or 60% epiboly stages.

Cloning and sequencing of cth1 cDNA

Full length *cth1* was cloned from an oocyte stage cDNA library after subtractive hybridization screening (described in Stevens et al., 1996b). Sequencing was performed as in that publication. The sequence described here is available from the EMBL database under accession number Y10163.

Northern blot analysis

Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). 2 μg total RNA per lane were electrophoresed through a 1.2% agarose/formaldehyde gel, transferred onto a Nytran membrane (Schleicher and Schuell Nederland BV, the Netherlands) and vacuum-baked for one hour at 80°C. Hybridization with Dig-labeled RNA probe (same as for ISH) was performed overnight at 68°C in 5 x SSC/ 50% formamide/ 0.02% sodium dodecylsulphate/ 0.1% N-lauroylsarkosine/ 2% blocking reagent (Boehringer Mannheim (BM), Germany). Washes and chemiluminescent detection (CDP-star) were carried out as recommended by BM. Fuji X-ray films were exposed less than 1 minute to visualize *cth1* transcripts.

Whole mount In Situ Hybridization and cryo-sectioning

The ISH were carried out as in Stroband et al. (1995), with some modifications. Embryos were fixed in MEMFA (4% formaldehyde/ 0.1 M MOPS pH 7.4/ 2 mM EGTA/ 1 mM MgSO4), and washed in PBST (PBS buffer/ 0.1% Tween-20) before hybridizing. Methanol storage and proteinase K treatments were omitted. The *cth1* probe was diluted 2000 times in (pre)hybridization solution (50% formamide/ 2 x SSC/ 2% blocking reagent/ 0.1% Tween-20/ 5 mg/ml yeast RNA/ 50 μg/ml heparin) and hybridized at 60 - 70°C. After staining at room temperature, embryos were stored in 4% paraformaldehyde in PBS at 4°C. For photography, embryos were usually cleared in benzyl benzoate: benzyl alcohol (2:1). The staining is brown instead of blue, in non-cleared embryos (Figs. 4J-L).

Sections of whole-mount stained embryos were prepared by cryo-sectioning. Stained embryos were fixed in 4% paraformaldehyde/ 0.2% glutaraldehyde/ PBS, rinsed 3 times in PBST (5 minutes each) and mounted in 1.5% agarose/ 5% sucrose. Following overnight immersion with 30% sucrose at 4°C, agar-mounted embryos were frozen in liquid nitrogen and then sectioned at -23°C.

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Chapter 6

C3H zinc fingers: mediators of developmental pluripotency?

Carine Stevens

In the Discussion of Chapter 5, we hypothesize that the cth1 gene might be involved in the prevention of determination events during early development. Two recent papers (Mello et al., 1996; Seydoux et al., 1996) led us to shortly review the literature on the C3H class of genes, to which the cth1 gene belongs.

Introduction

Composing an adult organism from a fertilized oocyte is a complex process reflecting many developmental decisions. Totipotent zygote cells enter distinct pathways to differentiate into specialized cells of the adult body. The choice for a particular cell lineage is accompanied by stepwise restriction of cellular potential and, ultimately, commitment of cell fate. Distinct cell lineages have specific timing of specification events. While certain cells undergo determination processes early, others temporarily maintain their developmental potency and remain unspecified until later developmental stages. Cells of the germ line even remain totipotent all throughout development and adult life.

A mechanism for restricting potency involves inactivation of gene expression by DNA methylation (Brandeis et al., 1993). Whether methylation is also used to silence differentiation genes in early development is not yet clear. Recently, exciting publications pointed at a novel class of zinc finger proteins as regulators of cellular potency. The finger motif of this class of genes (Fig. 1) contains three cysteines and one histidine and shall here be referred to as C3H. C3H class genes were identified in several species (Worthington et al., 1996, and below). In mammals for example, they are present as the *TIS11* family of primary response genes to growth factors and tumor promoters (Herschman et al., 1994). Members of this family are considered regulators of gene expression. In general, their regulatory activity is of an inhibiting nature.

In *C. elegans* (Guedes and Priess, 1997; Seydoux et al., 1996; Mello et al., 1996) and in fish (this thesis and unpublished observations) C3H class genes are expressed in uncommitted cells of the early embryo. Together, these studies made us believe that these genes may be widely involved in preserving cellular potency in specification events during development.

C3H gene expression and cellular potency

In the nematode *Caenorhabditis elegans*, unequal divisions of the zygote produce germ line blastomeres from which totipotent germ cells descend. Germ line blastomeres remain totipotent because they do not respond to somatic differentiation signals. This indifference is most likely caused by the suppression of transcription in these blastomeres, and the concerted activities of two C3H class proteins, PIE-1 and MEX-1, are clearly involved in this (Guedes and Priess, 1997; Seydoux et al., 1996; Mello et al., 1996). PIE-1 activity suppresses the appearance of embryonic transcripts (Seydoux et al., 1996), while MEX-1 restricts PIE-1 activity solely to the germ line blastomeres (Guedes and Priess, 1997).

Our studies on RNA expression of the C3H class gene *cth1* in carp and zebrafish (unpublished results) led us to propose a role for the cth1 protein

in maintenance of cellular potential during two phases of early development (Chapter 5). Firstly, maternal *cth1* transcripts are present in all blastomeres and disappear gradually after the onset of transcription. Given the high degree of identity between the *cth1* finger motifs and those of the mammalian *TIS11* genes, the TIS11 properties (see Introduction), and the fact that fish blastomeres are not committed to a particular fate during early development (Kimmel and Warga, 1987), we proposed that maternally-derived *cth1* protects the cellular potential of early blastomeres, by preventing the translation of certain target mRNA(s), which may otherwise trigger inductive events. Secondly, future mesendoderm cells specifically express zygotic *cth1*. These cells are not committed to their respective fates until midgastrulation (Ho and Kimmel, 1993) and the *cth1* expression may help them to maintain their uncommitted state.

Data concerning embryonic expression of C3H class genes in other species leave room for an interpretation towards maintenance of cellular potency. In *Drosophila*, the developmental expression of three C3H class genes has been studied. *Drosophila DTIS11* (Ma et al., 1994), a sequence with highest identity to the murine *TIS11B*, is maternally and zygotically transcribed. The maternal message is ubiquitously distributed and succeeded by zygotically expressed message. After the cellular blastoderm is formed, the overall *DTIS11* expression-level decreases. *Unkempt* (Mohler et al., 1992), a distant member of the C3H class having five finger motifs, is also present as a maternal transcript in early *Drosophila* embryos, like *DTIS11*. Upon gastrulation the *unkempt* mRNA level is increased in invaginating tissues (prospective mesoderm, endoderm and cephalic fold). Although *unkempt* expression is temporarily upregulated in neural tissues, the expression patterns of *unkempt, DTIS11* and fish *cth1* bear similarities, suggesting analogy of function.

Northern blotting demonstrated that *Suppressor of sable (sus)*, another *Drosophila* C3H gene, is expressed throughout life (Voelker et al., 1991). Sus protein is thought to be involved in RNA splicing. However, studies dealing with this property did not recognize the C3H motif and RNA binding was attributed to other motifs. Of a fourth *Drosophila TIS11* gene, *TIS11cc1* (largely similar to *DTIS11*) only the sequence is known (Warbrick and Glover, 1994).

In adult species, C3H genes are widely expressed (Northern blot analysis by Tippetts et al., 1988; Ino et al., 1995). Though most cells are restricted and differentiated in adult tissues, stem cell populations continue to produce cells that have not undergone terminal differentiation yet. It is possible that in adult tissues, C3H expression is restricted to such populations, which would also point to an involvement in cellular

A

		finger 1	spacing	finger 2
		▼ ▼ ▼ ▼		▼ ▼ ▼ ▼
mouse	TIS11a (P22893)	RYKTELCRTYSESGRCRYGAKCQFAH	GLGELRQANRHP	KYKTELCHKFYLQGRCPYGSRCHFIH
human	TIS11a (P26651)	RYKTELCRTFSESGRCRYGAKCQFAH	GLGELRQANRHP	KYKTELCHKFYLQGRCPYGSRCHFIH
rat	TIS11a (JC1255)	RYKTELCRTYSESGRCRYGAKCQFAH	GPGELRQANRHP	KYKTELCHKFYLQGRCPYGSRCHFIH
cow	TIS11a (L42319)	RYKTELCRTFSESGRCRYGAKCQFAH	GLGELRQASRHP	KYKTELCHKFYLQGRCPYGSRCHFIH
mouse	TIS11b (P23950)	RYKTELCRPFEENGACKYGDKCQFAH	GIHELRSLTRHP	KYKTELCRTFHTIGFCPYGPRCHFIH
human	TIS11b (Q07352)	RYKTELCRPFEENGACKYGDKCQFAH	GIHELRSLTRHP	KYKTELCRTFHTIGFCPYGPRCHFIH
rat	TIS11b (P17431)	RYKTELCRPFEENGACKYGDKCQFAH	GIHELRSLTRHP	KYKTELCRTFHTIGFCPYGPRCHFIH
fruitfly	DTIS11 (U13397)	RYKTELCRPFEEAGECKYGEKCQFAH	GSHELRNVHRHP	KYKTEYCRTFHSVGFCPYGPRCHFVH
mouse	TIS11d (P23949)	RYKTELCRPFEESGTCKYGEKCQFAH	GFHELRSLTRHP	KYKTELCRTFHTIGFCPYGPRCHFIH
human	TIS11d (U07802)	RYKTELCRPFEESGTCKYGEKCQFAH	GFHELRSLTRHP	KYKTELCRTFHTIGFCPYGPRCHFIH
		******** * * * ** ****** * *** *** ******* * * **** **** *		

B

		finger 1	spacing	finger 2
		▼ ▼ ▼ ▼		▼ ▼ ▼ ▼
mouse	TIS11a (P22893)	RYKTELCRTYSESGRCRYGAKCQFAH	(x)12	KYKTELCHKFYLQGRCPYGSRCHFIH
carp	cth1 (Y10163)	RYKTELCSRYAETGTCKYAERCQFAH	(x)12	KYKTELCRTYHTAGYCVYGTRCLFVH
baker's yeast	CTH1 (P47976)	LYKTELCESFTIKGYCKYGNKCQFAH	(x)12	NYRTKPCINWSKLGYCPYGKRCCFKH
baker's yeast	CTH2 (P47977)	LYKTELCESFTLKGSCPYGSKCQFAH	(x)12	NFRTKPCVNWEKLGYCPYGRRCCFKH
fission yeast	ZFS1 (P47979)	LYKTEPCKNWQISGTCRYGSKCQFAH	(x)12	KYKSERCRSFMNYGYCPYGLRCCFLH
C. elegans	PIE-1 U62896)	EYKTRLCDAFRREGYCPYNDNCTYAH	(x)60	SNRRQICHNFER-GNCRYGPRCRFIH
C. elegans	MEX-1 (U81043)	AFKTALCDAFKRSGSCPYGEACRFAH	(x)17	KYKTQLCDKFSNFGQCPYGPRCQFIH
fruitfly	sus (M57889)	PRKLELCKFYLM-DCCAKRDKCSYMH	(-)	--KEFPCKYYYLGMDCYAGDDCLFYH
consensus		xxKTxL**C**xxxxxxx**G**x**C**x**Y**xxx**C**x**FAH**		x**Y**Kxxx**C**xxxxxxx**G**x**C**x**Y**G**x**R**C**x**F**x**H**
		L D K YM		NR M A D

		▼ ▼ ▼ ▼	
fruitfly unkempt (Z11527)	finger 1	YSADNYC------TKYDETT-GI-CPE-GDECP-YLH	(x)13
	finger 2	YYKTCMC-------VHDTDSRGY-CVKNGLHCA-FAH	(x)55
	finger 3	NYKTEPC------KR--PPRL---CRQ-GYACPQY-H	(x)12
	finger 4	KYRSTPCPNVKHGEEWGEP--GN-CEA-GDNCQ-YCH	(x)10
	finger 5	IYKSTKC------NDVQQA--GY-CPRS-VFCA-FAH	

Fig. 1 Alignments of Cys-three-His domains. **A** Comparison of the C3H domains of TIS11 zinc finger proteins. Cysteine and histidine residues of the putative zinc fingers are indicated with ▾ symbols. Note that the TIS11 proteins have identical numbers of amino acids between the two fingers. The mammalian proteins have been classified in three groups: TIS11a, TIS11b and TIS11d. TIS11a proteins have also been reported as TTP, TIS11 and Nup475. The mammalian TIS11 finger domains and their spacing are highly conserved. The *Drosophila* DMTIScc1 sequence is omitted for its C3H domains are identical to those of *Drosophila* DTIS11. Asterisks represent conserved amino acids. **B** Comparison of TIS11 and other C3H domains. Mouse TIS11a represents TIS11 family members. The carp and yeast C3H domains and their spacing show larger degrees of homology to the TIS11 family than the *C. elegans* and fruitfly sequences. TIS11 family domains are separated by 12 amino acids whereas the more distantly related domains of PIE-1, MEX-1, sus and unkempt are differently spaced. *Drosophila* unkempt has five zinc finger domains. The lengths of these domains are not conserved. To provide optimal alignment of the unkempt residues, gaps were introduced as Worthington et al. (1996). Dashes represent gaps and x represents any amino acid.

potency. Two examples are described below.

Anomalies in immune response were demonstrated in mice that are deficient for the TTP protein (also known as TIS11, TIS11a and Nup475). These mice, generated by gene targeting, seem normal at birth but soon developed abnormalities in neonatal life, such as cachexia, inflammations and myeloid hyperplasia (Taylor et al., 1996a). The symptoms were undone by administrating monoclonal antibodies to TNFα (tumor necrosis factor α), a cytokine implicated in the activation of the immune system. These data demonstrate a role for TTP in the regulation of the immune response through negative modulation of TNFα. This regulation could involve interference in the activation of resting lymphocytes.

Human *TIS11d* has been associated with leukemogenesis. In a patient that suffered from acute T cell leukemia, the C-terminal portion and 3' untranslated region (UTR) of the *TIS11d* gene appeared to be replaced by that of an inserted DNA fragment (Ino et al., 1995). It was argued that chimeric TIS11d played a role in developing this leukemia and that *TIS11d* is a potential oncogene. The 3'UTRs of *TIS11* genes (including carp *cth1*) usually contain motifs that are thought to confer instability to the mRNA transcript. Ino and colleagues suggested that the 3'UTR replacement caused stabilization of transcripts and thereby a higher expression-level than normally occurs. If C3H motifs prevent differentiation, overexpression of this hybrid protein might arrest lymphoid cells in a proliferative phase instead of undergoing differentiation, perhaps resulting in cancer.

Role of the C3H zinc finger

If an unambiguous role for C3H proteins exists, it is reasonable to assume that this role is linked to the finger motif. Observations on *TIS11* in cell culture studies led to the general belief that C3H motifs are novel nucleic acid binding domains involved in regulation of gene expression (Herschman et al., 1994). However, conclusive evidence for the functionality of the finger remains to be given. Worthington and colleagues (Worthington et al., 1996) demonstrated zinc binding by the C3H motifs of mouse TTP and proposed a structure for the finger. Clearly, studies resolving this issue are essential for an understanding of these proteins. Affecting regulation of gene expression likely involves meddling in transcription or translation. Information as to the biochemical properties of the motif does not point to one single mechanism. A role for PIE-1 in transcriptional regulation is plausible, for the protein resides predominantly in the nucleus and represses embryonic transcription (Seydoux et al., 1996). Even though the molecular nature of MEX-1 function is not known yet (Guedes and Priess, 1997), it is apparent that a transcriptional role is not to be expected for this protein since it is located in the cytoplasm.

Similarly, a role in translation seems most plausible for the encoded carp cth1 protein as the sequence lacks an obvious nuclear localization signal, and maternal expression largely precedes transcriptional activity of the embryo (Chapter 5). These observations suggest that regulation occurs at both levels and by different proteins. It is also possible that a given C3H finger has binding affinities to both RNA and DNA, as is shown for the TFIIIA C2H2 zinc finger protein (Theunissen et al., 1992). The ultimate role of a C3H protein could then depend on its location in the cell. Studies on the TTP protein are indicative of a complicated regulation of subcellular location. Taylor and colleagues (1996b) demonstrated that mitogen-mediated stimulation caused rapid translocation of this protein from the nucleus to the cytosol. Though the authors discussed a transciptional role, the presented data do not exclude a role in translation as well.

In conclusion

Having been identified from fungi to mouse, the C3H motif has certainly proven a widespread distribution. Sequence conservation over such evolutionary distances often indicates an important function. We propose a role in the maintenance of developmental potential in ontogenesis, and possibly in stem cell populations. Several levels of decision-making during ongoing restriction of developmental potential require the assurance of potential fates of cells. The C3H family of zinc finger proteins may provide candidates to fulfill such tasks. Regardless of the eventual role of the C3H motif, the study of this novel class of genes will undoubtedly provide interesting insights into developmental mechanisms.

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Chapter 7

General Discussion

Introduction

For the isolation of developmental genes, several different molecular cloning strategies are applied. For example homology cloning, one of the technically most straightforward methods, is used to isolate homologs of genes proven to be important for development in other species. Many developmental genes were cloned for *Drosophila* and, by homology cloning, a great deal of these were shown to have counterparts in vertebrates (Nüsslein-Volhard, 1996). However, *Drosophila* is an invertebrate and lacks some of the features of the vertebrate body, for example the notochord and neural crest (Driever and Fishman, 1996). Therefore, it is of importance to also undertake searches after those genes that specifically regulate vertebrate development. Differential screening of a subtracted library and differential display respectively yielded *eomes* and *antipod* (Ryan et al., 1996; Stennard et al., 1996), novel genes of *Xenopus*. For fish in particular, the molecular isolation and characterization of novel developmental genes may benefit the large-scale mutagenesis screens of zebrafish that were recently performed (Driever and Fishman, 1996; Haffter et al., 1996). Over 400 genes were phenotypically identified (Schier et al., 1996), but only a few mutations are molecularly characterized yet (for example *no tail,* Schulte-Merker et al., 1994; *floating head*, Talbot et al., 1995). An important strategy for the cloning of mutated genes is the candidate gene approach (Postlethwait and Talbot, 1997; Schier et al., 1996). A candidate gene is selected on the basis of its expression pattern, and subsequently its location on the zebrafish genetic map is compared to the location of the mutation. At present, the number of genes for which sequences are known is a limiting factor in the large scale application of this approach (Postlethwait and Talbot, 1997; Schier et al., 1996). Especially the subtractive hybridization strategy as performed in this thesis (see sections: 'Subtractive hybridization' and 'Differentially expressed genes') may provide candidate genes.

Two strategies

At the time that the investigations of this thesis were initiated, only few developmental genes had been cloned for fish. We set out to identify genes that relate to issues in the center of attention of our research group and several other groups. Our interest focused on the inductive processes that take place prior to and during gastrulation, in particular concerning mesoderm and endoderm formation and differentiation, and formation and patterning of the anteroposterior and dorsoventral axes. We chose two molecular strategies to identify genes related to these issues.

Firstly, a homology-based cloning strategy was applied to specifically isolate members of the superfamily of homeobox-containing genes (Chapters 2 and 3), both clustered and non-clustered homeobox genes. In

addition to the homeobox screening, a subtractive hybridization screening was performed to isolate genes on the basis of their differential expression between two developmental stages (described in Chapter 4). These two stages are the oocyte and the early segmentation stages and were selected because they relate to early inductive processes.

Screening for homeobox genes

The screening of the segmentation stage cDNA library with a mixture of homeobox sequences as probe yielded 26 positive clones (Chapter 2) of which two, representing *carp-cdx1* and *Hoxb-1*, are described in Chapters 2 and 3 of this thesis respectively. The remaining clones are described elsewhere (*Hoxb-3*, In Der Rieden et al., 1996; *carp-ovx1*, Stroband et al., 1996a) or are still under examination (for example *Hoxc-6*, Te Kronnie unpublished results).

Hoxb-1, located at the 3' end of the genomic *Hox* cluster, is one of the first *Hox* genes expressed in the embryo (Krumlauf et al., 1993) and its anterior expression limit reaches into the anteriormost expression domain of *Hox* genes: the hindbrain. *Hoxb-1* genes have been described for a number of vertebrate classes (*Xenopus*, Godsave et al., 1994; mouse, Murphy et al., 1989; human, Acampora et al., 1989; chicken, Sundin et al., 1990). Chapter 3 provides a description of the full protein coding sequence and mRNA expression pattern of this gene in teleosts, for which heretofore only the 5' and 3' non-coding regions had been identified (pufferfish, Marshall et al., 1994; Pöpperl et al., 1995). Carp *Hoxb-1* expression, strongest in the anterior part, stretches from the boundary between the future rhombomeres (r) 3 and 4 towards the posterior end of the embryo. During the segmentation period the hindbrain expression is, as in other vertebrates, restricted to r4 (see references above) where its functional domain lies (Maconochie et al., 1996). The role of *Hoxb-1* is that of maintenance of the segmental identity of r4, in particular of neurons of the VIIth nerve (Studer et al., 1996; Goddard et al., 1996). The restricted expression in r4 is a useful marker of r4 and hindbrain, and has been used as such in the analysis of the mRNA expression pattern of the carp *Hoxb-3* gene, which appeared as one of the other positive clones resulting from the screen for homeobox genes (In Der Rieden et al., 1996). Neural crest cells migrating from r4 by the stage of 15 somites also express *Hoxb-1*. This expression is currently being used as a marker of these cells in transplantation studies (personal communication Te Kronnie).

In general, neurectodermal expression of *Hox* genes receives more attention than their expression in mesoderm and endoderm. For *Hoxb-1* the earliest expression is in fact higher in the hypoblast, the future mesoderm and endoderm, than in the epiblast (future ectoderm and neuroderm) and the gene continues to be expressed in the mesoderm until at least the end of the segmentation period. As in the neurectoderm, the

Hox expression in mesoderm and endoderm may also be important for anteroposterior specification.

The second clone derived from the screen for *Hox* genes represents the *carp-cdx1* gene (Chapter 2), a non-clustered member of the superfamily of homeobox genes, belonging to the *caudal* (*cad*) class (Bürglin, 1994). The expression of this gene is a distinct marker of (ventro)posterior regions. Early transcription of *cdx1*, during early epiboly, is found on the ventral side of the embryo in cells near the margin of the blastoderm. As epiboly proceeds, the expression moves towards posterior regions of the egg, meanwhile also spreading to dorsal regions of the margin. Initially restricted to the outer cell layer of the blastoderm, transcripts are also present in the hypoblast after midgastrulation.

The region-specific *cdx1* expression was used as a marker in experiments undertaken in our group to examine the degree of mesoderm differentiation, and formation of dorsoventral and anteroposterior polarity in blastoderms from which the yolk cell had been removed and, as a consequence, lacked inductive signals that are expected to be transferred from this cell to the blastoderm (Stroband et al., 1996b; Bozhkova et al., 1994; Te Kronnie and Stroband, 1996). These studies indicated that blastoderms that were explanted after formation of the Yolk Syncytial Layer (YSL), at the tenth cleavage, are capable of mesoderm differentiation (Bozhkova et al., 1994). The comparison of expression of the *cdx1* gene, to indicate ventral and/or posterior regions, and a marker of dorsal (*gsc*; Stachel et al., 1993; Schulte-Merker et al., 1994) showed that already after the tenth cleavage the blastoderm has received information necessary to realize dorsoventral polarity. In blastoderms explanted one hour later (at sphere stage), the spatial organization of *cdx1* and *gsc* expression pointed out that anteroposterior axis formation was taking place. Clearly, regulative interactions between yolk cell and blastoderm from the tenth cleavage to the sphere stage, are necessary for gastrulation movements and anteroposterior axis formation to be initiated (Te Kronnie and Stroband, 1996).

Not only are *cdx* genes markers of posterior position in the embryo, studies in several species suggest a role for these genes in anteroposterior patterning of the embryonic axis (reviewed in McGinnis and Krumlauf, 1992; Griffin et al., 1995; Pownall et al., 1996; Subramanian et al., 1995, and references therein). The development of the trunk and tail requires FGF signaling (Amaya et al., 1993; Griffin et al., 1995; Pownall et al., 1996). It has been demonstrated in *Xenopus* that posterior *Hox* genes, which are important for anteroposterior patterning (Krumlauf, 1994), depend on FGF signaling for their expression (Pownall et al., 1996). *Cdx* genes may fulfill an important role herein as they are thought to act downstream of FGF signaling and are expected to directly activate the

expression of posterior *Hox* genes (Subramanian et al., 1995; Pownall et al., 1996), thereby promoting posterior development.

Subtractive hybridization

When the work described in Chapter 4 was initiated, a number of strategies were generally employed for isolation of differentially expressed genes (Sargent and Dawid, 1983; Rebagliati et al., 1985; Sargent, 1987; Almendral et al., 1988; Wang and Brown, 1991). We have applied a simplified modification of a subtractive hybridization method introduced by Wang and Brown (1991, 1993) which they used to isolate genes involved in amphibian tail resorption (Wang and Brown, 1993; Brown et al., 1996). Of the two stages we selected for subtraction, the oocyte stage and the early segmentation stage, enriched probes were made (see section: 'Two strategies' and Chapter 4) and used for differential screening (Almendral et al., 1988).

The advantage of using enriched probes lies in the ease of detecting positive clones. In a previous differential screen of the early segmentation stage (unpublished results) we used probes generated by the same protocol, but without enrichment. However, the selection of positive clones using non-enriched probes is difficult and time-consuming. In the process of enrichment (subtractive hybridizations), the ubiquitously present transcripts are largely removed, thereby greatly increasing the fraction of differentially expressed mRNAs in the probe mixture, resulting in stronger hybridization signals and improved detection of the corresponding clones. Though, technically, the subtractive hybridization is rather challenging, it is worth investing the effort for large scale screens as it facilitates and improves the detection of positive clones, even those representing genes expressed at low levels.

Differentially expressed genes

After a differential screen, those cDNA clones were selected ('positive') that hybridized to the probes of one of the two stages and not to those of the other stage. Using this criterion, a few dozens of clones were selected (Chapter 4), of which at least 50 percent appeared to represent differentially expressed genes, as checked by In Situ Hybridization (ISH). We expect that this percentage could easily be increased in future screens, if some adjustments are made in the strategy. In Chapter 4 it is described how the subtractive hybridization strategy yielded 15 oocyte stage clones, representing differentially expressed genes, and 26 clones from the early segmentation stage. Of these differentially expressed genes, 27 in total were considered novel genes as they were not homologous to sequences in the international sequence databases (for example EMBL) and were thus potential candidates for further study. We chose to focus on genes that are locally expressed in the embryo (local

expression), i.e. restricted to specific regions of the embryo, in addition to their differential expression during development. Though ubiquitous transcription does not preclude a gene from having an important developmental function, it excludes the mRNA expression from being used as marker. Clear examples hereof are the DVR-1 and ß-catenin proteins in zebrafish that have important functions in mesoderm induction and dorsal signaling respectively, but whose mRNAs are ubiquitously present throughout the embryo (Schneider et al., 1996; Kelly et al., 1995; Helde and Grunwald, 1993).

Segmentation stage cDNA clones

Seventeen of the 26 cDNA clones from the early segmentation stage represented locally expressed genes, as listed in Chapter 4. Of all these genes, we were interested in two clones in particular: 3-28 and 3-19. 3-28 RNA was found in a gradient from posterior to anterior in the segmentation stage embryo (Chapter 4), resembling but not identical to *cdx1*. Clearly, 3-28 RNA is of use as marker of posterior. Analysis of the sequence of this clone did not reveal homology to known genes, suggesting it represents a novel gene. Also, we were unable to discern a putative protein coding region. Northern blotting showed that the clone contains a partial sequence belonging to a transcript of approximately 4 kb length. Taken together, we suppose that the sequence in clone 3-28 is (part of) the 3'UTR of a larger transcript. Further attempts to isolate the full length sequence of this transcript were not successful yet. Information about the possible function of an unknown gene is usually gained by examining the protein sequence and the possible functional motifs within it, or by performing functional studies (for example overexpression or depletion of mRNA). Hence it is necessary to clone the full protein coding sequence and, preferably, also the UTRs. As a protein coding region was not available for clone 3-28, we were unable to further explore the features of the gene in question. For similar reasons we did not further pursue clone 3-19, that recognized transcripts of a novel gene expressed in neurectoderm and mesendoderm at the stage of 15 somites, axial and paraxial mesoderm excluded.

Oocyte stage cDNA clones

Of the 15 stage-specifically expressed genes isolated from the oocyte stage, 11 were locally expressed in the embryo, after the cleavage stages (Chapter 4). Three cDNA clones represented one gene, *cth1*, which seemed to be important for formation of mesoderm and endoderm and was selected for further study. This gene will be discussed later in this chapter. The remaining eight clones were derived from mitochondrial 16s ribosomal RNA sequences. Although 16s rRNAs belong to the housekeeping RNAs and are no key regulators of development, they are

specific markers of mitochondria. In *Xenopus*, mitochondria are translocated to the dorsal side during the first cell cycle (Yost et al., 1995). Hence, 16s rRNA transcripts mark dorsal in *Xenopus*. We are currently investigating, using our carp 16s rRNA probes, whether dorsal translocation of mitochondria during cleavage stages occurs in the teleost fish as well. So far, the earliest molecular indication of the dorsal side in teleost embryos is the ß-catenin translocation into dorsal nuclei, at the high blastula stage (see Chapter 1, section: 'Molecular nature of inductive signals' and Schneider et al., 1996).

Our studies and those of other groups interested in how initial dorsoventral and radial asymmetries are established, have found no evidence so far of non-homogeneously distributed messenger RNAs in early blastomeres. All our oocyte stage derived clones showed equal blastomeric distribution of transcripts with respect to radial or dorsoventral asymmetries. It is possible that the early asymmetries in the fish egg are not the result of differential distribution of mRNA transcripts in the preMBT embryo, but may be caused by regional translational differences within the embryo, or by post-translational modifications instead. Translational regulation is clearly essential for early development, and can occur by general mechanisms (i.e. masking from translation by RNA binding proteins) or sequence specific mechanisms (depending on specific sequences in the mRNA 3'UTR) (reviewed in Hake and Richter, 1997).

The post-transcriptional regulation of *DVR-1* and *ß-catenin* activities in zebrafish are examples in support of this notion. The *DVR-1* gene is the zebrafish homolog of *Xenopus Vg1* (Helde and Grunwald, 1993), a potent inducer of dorsal mesoderm whose transcripts are located at the vegetal pole of the *Xenopus* egg (Weeks and Melton, 1987; Kessler and Melton, 1995). In the zebrafish, *DVR-1* mRNA is homogeneously distributed (Helde and Grunwald, 1993). *ß-Catenin* maternal mRNA is present in all zebrafish blastomeres as well (Kelly et al., 1995). *ß-Catenin* and *DVR-1/Vg1* activities are controlled at the protein level. ß-catenin function involves nuclear translocation (Schneider et al., 1996; Larabell et al., 1997). DVR-1/Vg1 protein precursors need to be processed to functional proteins in order to acquire mesoderm inducing activity (Dohrmann et al., 1996). It is possible that in fish early asymmetries arise mainly at the level of the (functional) protein, not at the level of mRNA distribution.

Cth1

Chapter 5 deals with the RNA distribution and role of *cth1*, a novel gene expressed in (pre-)gastrulating carp embryos. The expression pattern of the gene suggests a role in mesoderm and endoderm formation; its transcripts, after being present in all blastomeres as maternal mRNAs, reappear in margin cells destined to become mesoderm and/or endoderm.

We expect that *cth1* activity influences the formation and or differentiation of these tissues. Taken together, 1) the expression pattern, 2) the presence of two putative C3H zinc fingers (Worthington et al., 1996) in the protein coding sequence and 3) relationship to the mammalian *TIS11* family of primary response genes to growth factors and tumor promoters (Herschman et al., 1994), led us to suggest a role for the *cth1* gene (Chapters 5 and 6). We proposed that this role could be one of (temporary) maintenance of the cellular potential. The inhibition of the translation of distinct target mRNAs, through binding of *cth1*'s putative zinc fingers to *cth1* response elements (CREs) in target genes, could prevent or delay the selection of certain differentiation pathways.

For cells at the margin of the blastoderm, it may be particularly useful to have the disposal of a mechanism that aids them to respond to the expression of genes at a specific stage of development. Mesoderm and/or endoderm inducing signals are transferred from the yolk cell cytoplasm to cells in the margin of the blastoderm (Mizuno et al., 1996; Stroband et al., 1996b; Grunwald et al., 1996), and from MBT onwards induce cells to activate gene expression. However, this induction may be rather unsubtle, in that cells may be induced to activate the expression of genes that are not necessarily or not yet required, just like animal cap cells express mesoderm-specific genes upon treatment with mesoderm inducing substances *in vitro* (for example Oda et al., 1995). Thus, cells may not (yet) be capable of selectively activating the transcription of solely the genes required at *that* moment of development, but activate an abundance of genes instead. Indeed, margin cells express a multitude of genes among which are *ntl*, *gsc*, *lim1* and *cdx1* (Schulte-Merker et al., 1992; Stachel et al., 1993; Toyama et al., 1995; Joly et al., 1992; also see Chapter 1). By repressing the translation of certain mRNAs, *cth1* (and perhaps related genes) may provide a secondary mechanism to adjust a cell's gene expression to its needs. How *cth1* activity could affect a cell's fate will be explained below by the hypothetical example of *cdx1* mRNA being translationally repressed by *cth1*.

Caudal (*cad*) class family members promote posterior development, by regulating the expression of posterior *Hox* genes (Pownall et al., 1996; Subramanian et al., 1995; Mlodzik et al., 1990; MacDonald and Struhl, 1986, and discussed above). In carp and zebrafish, *cdx1* was expected to fulfill a role in posterior development as well (see Chapter 2; Joly et al., 1992). The gene is initially expressed in ventrolateral cells of the margin and extends to all around the margin in the posterior region of the embryo during late gastrulation. In *Drosophila*, the protein expression pattern of *cad* is a modified version of the initial RNA expression pattern, as *cad* mRNA in the anterior part of the fruitfly embryo is translationally repressed by bicoid protein (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). The expression of *cdx1*, promoting posterior development, already during

early epiboly stages may be premature. *Cth1* could suppress the translation of these early *cdx1* transcripts and thus protect these cells from acquiring posterior features too early.

Whether *cth1* actually affects the translation of *cdx1* mRNA remains to be examined. In light of the proposed role for *cth1* one can imagine that the gene is involved in the processes that lead to the commitment of margin cells to a hypoblast-derived fate (mesoderm or endoderm, see Chapters 5 and 6). Embryonic *cth1* mRNA is present from the beginning of epiboly until approximately midgastrulation, in cells that are positioned at the blastoderm margin until 50% epiboly and involute immediately upon the onset of gastrulation. The inductive signaling leading to formation of hypoblast tissues occurs early in development (Bozhkova et al., 1994), but cells are not committed to a hypoblast-derived fate before midgastrulation (Ho and Kimmel, 1993). Up to approximately this stage, hypoblast cells possess *cth1* transcripts. It is tempting to speculate that the disappearance of *cth1* transcripts (and assumedly proteins) is correlated to the event of commitment.

Notably, only the early involuting hypoblast cells, that have the choice of either the mesodermal or the endodermal fate (Kimmel et al., 1990; Kimmel et al., 1995) express *cth1* whereas later involuting cells, only giving rise to mesoderm, do not. Fleig (1990) and Van Gestel et al. (1997) put forward the idea that different migration pathways (i.e. along the YSL or along the epiblast inner surface) exist for cells of the hypoblast and that these may affect their commitment to either the endodermal or the mesodermal fate. If migration pathways determine fate, then hypoblast cells will need to have the appropriate cell surface molecules available to bind to the substrate. That hypoblast cells express hypoblast-specific cell adhesion molecules is proposed by Ho and Kimmel (1993) who observed that only hypoblast cells that were transplanted to ectopic sites (epiblast) after midgastrulation had acquired the capacity to reenter the hypoblast. Prior to midgastrulation, such molecules could be translationally suppressed by (genes like) cth1.

Additional remarks and suggestions for future research on cth1

Functional studies in support of the proposed role for the cth1 protein are laborious and were for that reason, unfortunately, beyond the potential of this thesis. We have assumed that the pattern of protein expression closely follows that of its RNA expression pattern, a cth1 specific antibody will allow to explore this assumption. Also, zinc finger identity of the C3H motifs in this class of proteins needs further examination, currently undertaken by Worthington and colleagues (mouse TTP/Nup475; Worthington et al., 1996). Furthermore, whether cth1 brings about the translational repression of target mRNAs is unknown, and neither is the

identity of these target mRNAs known. According to its proposed role, the loss of function of *cth1* in embryonic development may effectuate premature cell differentiation. Loss of function may be mimicked by the depletion of the endogenous *cth1* mRNA pool by microinjection of antisense oligonucleotides to *cth1* (see Heasman et al., 1992). Overexpression of a constitutively expressed *cth1* construct would be expected to have opposite effects.

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Summary

The formation of germ layers during gastrulation and the specification and patterning of the body axes are important events in the development of the embryo. The investigations described in this thesis aimed to isolate and characterize the distribution of transcripts of genes, in particular *novel* genes, that are expressed during the formation of mesoderm and the patterning of the anteroposterior axis in the carp (*Cyprinus carpio*), a cyprinid teleost fish. Such studies may contribute to a better insight in the molecular mechanisms underlying the above processes, in two respects. Firstly, the characterization of a gene's expression pattern is one of the first steps towards the elucidation of its function. Especially the characterization of novel genes may provide a key to new insights in development. Furthermore, in studies of development, it is of importance to have markers that identify specific cell types, for example early mesodermal precursor cells. The isolation of genes that are specifically expressed in certain cell types provides such markers.

Two molecular approaches were chosen for gene isolation. Firstly, we specifically searched for homeobox genes, which encode transcription factors with important regulatory functions during development. A particular class of homeobox genes, the *Hox* genes, provides cells along the anteroposterior axis with positional information and the expression patterns of members of this class are excellent markers of position on this axis. Our second approach was a subtractive hybridization strategy. It was applied to isolate genes that are differentially expressed between the oocyte and the early segmentation stage, a period during which mesoderm is induced.

For the identification of homeobox-containing sequences in a carp early segmentation stage cDNA library, we used a probe that was composed of a mixture of homeobox fragments, produced by PCR. The PCR primers were designed against the most conserved regions of the homeobox. This approach yielded a number of different genes of which two are described in this thesis. The gene *cdx1* (**Chapter 2**) is a member of the *caudal* class of homeobox genes and is expressed in ventrolateral cells of the embryo prior to gastrulation. During gastrulation, transcripts of this gene accumulate in the posterior half of the embryo. The functions of *caudal* class genes of *Drosophila*, mouse and *Xenopus* indicate that genes of this class mediate the specification of posterior positional values in the embryo. Because of their characteristic distribution, *cdx1* transcripts are useful markers of (ventro)posterior position in the embryo and have been used as such in the studies of mesoderm formation and anteroposterior patterning in blastoderm explants, performed in our laboratory.

A second gene isolated in the search for homeobox genes was the *Hoxb-1* gene, described in **Chapter 3.** This gene belongs to the class of *Hox* genes, whose members are organized into clusters in the genome of

many species. With expression reaching into the hindbrain, the *Hoxb-1* gene is one of the most anteriorly expressed *Hox* genes. Its most prominent expression, especially during segmentation, is found in rhombomere 4. In the late segmentation stage embryo, *Hoxb-1* expression is a valuable marker of this rhombomere and the neural crest cells at that level of the hindbrain.

Chapter 4 describes the cloning of genes on the basis of their differential gene expression between the oocyte and the early segmentation stage, using a subtractive hybridization strategy. Fifteen genes, identified from the oocyte stage cDNA library, are expressed in early development, when mesoderm induction occurs, and their expression disappears before the beginning of segmentation. From the early segmentation stage cDNA library, 26 genes were selected whose expression was activated during segmentation but not yet in early development, coinciding with the differentiation of the mesoderm and the patterning of the anteroposterior axis. In total 27 genes appeared to code for novel proteins and are therefore candidates for further studies and may provide a better insight into molecular mechanisms underlying developmental processes. Also in light of the large scale mutagenesis screens of zebrafish that have recently been undertaken in a number of laboratories and for which the affected genes yet need to be molecularly identified, it is important that the search for novel genes continues for only few candidate sequences are available so far. The subtractive hybridization strategy described in this thesis appears a worthwhile technique to obtain such candidate sequences.

Further investigations of these novel genes were restricted to a detailed characterization of the expression of one gene: *cth1*. **Chapter 5** gives a description of the distribution of the mRNA transcripts of this gene during cleavage, blastula and gastrula stages. Whereas maternal *cth1* mRNA is ubiquitously distributed in the blastomeres, the embryonically transcribed *cth1* mRNA is expressed after the late blastula stage, in cells at the blastoderm margin which have mesodermal and endodermal fates. The *cth1* transcripts disappear around midgastrulation, coinciding with the commitment of cells to the mesodermal (or endodermal) fate. In the cth1 protein, motifs containing three cysteines and one histidine (C3H) are present that are most likely zinc fingers, structures involved in the regulation of expression of target genes. The *cth1* mRNA expression pattern and the gene's homology to the *TIS11* family, a family of primary response genes whose expression is activated after treatment with for example growth factors, suggest a function for the *cth1* gene of maintenance of the cellular potential in cells with mesodermal (and endodermal) fates, and in cells of cleavage stages. By inhibiting the expression of certain target genes, cth1 could prevent or delay the selection of certain differentiation pathways, such as for example the

commitment to a mesodermal fate before midgastrulation.

Proteins containing C3H motifs are expressed in a number of species. For example in *C. elegans*, the PIE-1 protein is required to keep germline blastomeres totipotent during early development, most likely by suppressing the transcription in these blastomeres. In **Chapter 6** the literature on the C3H class of proteins is shortly reviewed and the hypothesis is proposed that they may be widely involved in preserving cellular potency in specification events during development.

In **Chapter 7** the results presented in previous chapters are discussed, with emphasis on the proposed role for *cth1* and how its activity could affect the fate of the cells expressing this gene.

Samenvatting

De vorming van de kiembladen tijdens de gastrulatie en de specificatie en patroonvorming van de lichaamsassen zijn belangrijke gebeurtenissen in de ontwikkeling van het embryo. Het onderzoek beschreven in dit proefschrift was gericht op de isolatie en de karakterisatie van expressiepatronen van genen, in het bijzonder *nieuwe* genen, die tot expressie komen tijdens de vorming van het mesoderm, en de patroonvorming van de anteroposteriore (kop-staart) lichaamsas in de karper (*Cyprinus carpio*), een cyprinide beenvis. Studies als deze kunnen in twee opzichten bijdragen aan een beter inzicht in de moleculaire mechanismen die ten grondslag liggen aan de bovenstaande processen. Ten eerste is het karakteriseren van het expressiepatroon van een gen één van de eerste stappen naar het ophelderen van de genfunctie. Vooral het karakteriseren van nieuwe genen kan een sleutel zijn voor nieuwe inzichten in de ontwikkeling. Verder is het in studies naar de ontwikkeling van belang om markers te hebben die specifieke celtypes identificeren, bijvoorbeeld vroege voorlopers van mesodermcellen. Het isoleren van genen die specifiek tot expressie komen in bepaalde celtypen, verschaft zulke markers.

Twee moleculaire benaderingen werden gekozen voor het isoleren van genen. Ten eerste zochten we gericht naar homeoboxgenen, die coderen voor transcriptiefactoren met belangrijke regulerende functies tijdens de ontwikkeling. Een speciale klasse van homeoboxgenen, de *Hox*-genen, voorziet cellen op de anteroposteriore as van informatie omtrent hun positie (positionele informatie). De expressiepatronen van leden van deze klasse zijn zeer geschikte markers voor posities op deze as. Onze tweede benadering was een subtractieve hybridisatie strategie. Deze werd toegepast om genen te isoleren die differentieel tot expressie komen tussen het oocytstadium en het vroege segmentatiestadium, de periode waarin het mesoderm geïnduceerd wordt.

Voor de identificatie van homeobox-bevattende sequenties in een cDNA bank van een vroeg segmentatiestadium van de karper gebruikten we een probe bestaande uit een mengsel van homeoboxfragmenten. Deze probe werd geproduceerd door middel van PCR met primers die ontworpen waren tegen de meest geconserveerde gebieden van de homeobox. Deze benadering leverde een aantal verschillende genen op waarvan er twee beschreven staan in dit proefschrift. Het *cdx1*-gen (**Hoofdstuk 2**) is een lid van de *caudal*-klasse van homeoboxgenen en komt vóór de gastrulatie tot expressie in ventrolaterale cellen van het embryo. Gedurende de gastrulatie accumuleren de transcripten van dit gen in de posteriore helft van het embryo. De functies van *caudal*-klasse genen van *Drosophila* (de fruitvlieg), de muis en *Xenopus* (een klauwpad) geven aan dat genen van deze klasse de specificatie van de positionele waarden posterior in het embryo bemiddelen. Vanwege hun karakteristieke distributie zijn *cdx1*-

transcripten bruikbare markers voor (ventro)posterior positie in het embryo; ze zijn als zodanig gebruikt in studies in ons laboratorium naar mesodermvorming en anteroposteriore patroonvorming in blastoderm-explanten.

Een tweede gen dat tijdens het zoeken naar homeoboxgenen werd geïsoleerd was *Hoxb-1*, dat beschreven is in **Hoofdstuk 3**. Dit gen behoort tot de klasse van *Hox*-genen. Leden van deze klasse komen in veel diersoorten voor en zijn georganiseerd in clusters in het genoom. Met een expressiepatroon reikend tot in de achterhersenen is het *Hoxb-1* gen een van de meest anterior tot expressie komende *Hox*-genen. Zijn meest prominente expressie, vooral gedurende de segmentatie, is in rhombomeer 4. Tijdens het late segmentatiestadium is de *Hoxb-1* expressie een waardevolle marker van dit rhombomeer, en de neurale lijst op dat niveau van de achterhersenen.

Hoofdstuk 4 beschrijft het kloneren van genen op basis van hun differentiële gen-expressie tussen het oocytstadium en vroege segmentatiestadium, gebruik makend van een subtractieve hybridisatie strategie. Vijftien genen, geïdentificeerd in de cDNA bank van het oocytstadium, komen tot expressie tijdens de vroege ontwikkeling, wanneer de mesoderminductie plaatsvindt. Hun expressie verdwijnt voor het begin van de segmentatie. Uit de cDNA bank van het vroege segmentatiestadium werden 26 genen geselecteerd waarvan de expressie pas werd geactiveerd gedurende de segmentatie, samenvallend met de mesodermdifferentiatie en de patroonvorming van de anteroposteriore as. In totaal bleken 27 genen te coderen voor nieuwe eiwitten en deze zijn daarom kandidaten voor verdere studies. Deze genen kunnen mogelijk een beter inzicht verschaffen in de moleculaire mechanismen van ontwikkelingsprocessen. Ook in het licht van de grootschalige mutagenese-screens van de zebravis die recent zijn ondernomen in een aantal laboratoria, en waarvoor de aangetaste genen nog moleculair moeten worden geïdentificeerd, is het belangrijk dat het zoeken naar nieuwe genen doorgaat omdat tot nu toe slechts weinig kandidaatsequenties beschikbaar zijn. De subtractieve hybridisatie die in dit proefschrift is beschreven, lijkt een waardevolle techniek om toe te passen ter verkrijging van zulke sequenties.

Verder onderzoek naar deze nieuwe genen werd beperkt tot een gedetailleerde karakterisatie van de expressie van één gen: *cth1*. **Hoofdstuk 5** geeft een beschrijving van de distributie van de mRNA transcripten van dit gen gedurende de klievingen, het blastula- en gastrulastadium. Terwijl het maternale *cth1* alomtegenwoordig aanwezig is in de blastomeren komt het embryonaal afgeschreven *cth1* na het late blastulastadium tot expressie in cellen in de rand van het blastoderm, die mesoderm of endoderm zullen worden. De *cth1* transcripten verdwijnen rond midgastrulatie, samenvallend met de commitment van cellen tot een

mesodermale (of een endodermale) toekomst. In het cth1 eiwit zijn motieven aanwezig die drie cysteïnes en een histidine (C3H) bevatten. Deze C3H motieven zijn meest waarschijnlijk zink vingers, structuren die betrokken zijn bij de regulatie van de expressie van doelwitgenen. Vanwege het *cth1* mRNA expressiepatroon en de homologie van dit gen met de *TIS11* familie, een familie van primaire respons genen waarvan de expressie wordt geactiveerd na behandeling met bijvoorbeeld groeifactoren, stelden we een functie voor het *cth1*-gen voor van behoud van cellulair potentieel in cellen met een mesodermaal (of endodermaal) lot, en in cellen in het klievingsstadium. Door de expressie van bepaalde doelwitgenen te remmen zou het cth1 eiwit de selectie van bepaalde differentiatieroutes kunnen voorkomen of uitstellen, zoals bijvoorbeeld de commitment tot een mesodermaal lot vóór midgastrulatie.

Eiwitten met C3H motieven komen tot expressie in een aantal diersoorten. In *C. elegans* (de nematode) bijvoorbeeld is het PIE-1 eiwit vereist om de blastomeren van de kiembaan totipotent te houden tijdens de vroege ontwikkeling. Zeer waarschijnlijk gebeurt dit door de transcriptie in deze blastomeren te onderdrukken. In **Hoofdstuk 6** wordt een kort overzicht gegeven van de literatuur over de C3H-klasse van eiwitten. De hypothese wordt voorgesteld dat deze eiwitten betrokken zijn bij het behoud van cellulair potentieel tijdens specificatieprocessen in de vroege ontwikkeling.

In **Hoofdstuk 7** worden de resultaten uit vorige hoofdstukken besproken, met nadruk op de voorgestelde rol voor *cth1* en hoe diens activiteit het lot van de cellen die dit gen tot expressie brengen, zou kunnen beïnvloeden.

Nawoord

In de voorgaande honderdzesentwintig bladzijden van dit boekje is slechts aandacht besteed aan de wetenschappelijke aspecten van het werk tijdens mijn AIO periode. Gelukkig kan ik zeggen dat dit een te eenzijdig beeld heeft geschetst van de afgelopen jaren. Naast de "onderdompeling in de wetenschap" is het juist ook een periode geweest van werken binnen een ontzettend leuke groep mensen, de (ex)vakgroep Experimentele Diermorfologie en Celbiologie. Alle collega's: bedankt voor jullie interesse, hulp, gezelligheid, etc etc! Dankzij jullie was EDC als een tweede "thuis".

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Ook veel niet-EDCers hebben me geholpen. Wilna, en natuurlijk Sietze, onmisbaar als verzorgers van de karpers en als mee-injiceerders en afstrijkers. Immers, zonder eieren en sperma geen embryo's! Verder Theo Goosen van Erfelijkheidsleer, bedankt voor je advies over de subtractie en andere moleculaire technieken. Dr Zhou Wang, thank you for discussing the pitfalls of the subtraction technique and your hospitality during my visit to Baltimore. Dr David Grunwald, thanks for your hospitality, help, suggestions and interest in "my" gene, and life in general - i appreciate it a lot and i hope we will stay in touch.

Vooral ook buiten Zodiac zijn er mensen en dingen die hun invloed op dit proefschrift hebben gehad. Bijvoorbeeld ballet, een goede compensatie voor de eenzijdigheid van het denkend-bestaan en een belangrijk element in mijn leven. Carine, mijn naamgenote, dank je wel voor 10-jaar goede lessen en het voeden van mijn interesse in de dans. Paul "Daikstr", het was maar goed dat we zo'n stevige deksel op de vuilnisbak hadden in de Cort van der Lindenstraat. Dankje voor je vriendschap en belangstelling. Ook Sandra, Ton, Frans Willem (FDoubleU), Maarten (je wilde niet in het dankwoord dus ik zal je achteraan zetten), en vele anderen: heel heel hartelijk bedankt voor jullie niet aflatende belangstelling en vriendschap. En niet te vergeten de luisterende oortjes als ik weer eens stoom moest afblazen (hopelijk is er geen blijvende gehoorbeschadiging ontstaan).

Ma, pa en Domi; het wordt niet vaak uitgesproken en ik wou deze kans daartoe maar eens aangrijpen: bedankt dat jullie altijd voor me klaarstaan.

Carine

Curriculum vitae

Catharina Johanna Maria (Carine) Stevens werd in Groesbeek geboren op 30 april 1968. Na het behalen van haar VWO diploma aan de Nijmeegse Scholen Gemeenschap begon zij in 1986 met de studie Milieuhygiëne aan de Landbouwuniversiteit te Wageningen. In maart 1992 rondde zij deze studie af, met de afstudeervakken Ontwikkelings- en voortplantingsbiologie (vakgroep Experimentele Diermorfologie en Celbiologie), Biochemie (vakgroep Biochemie) en een stage Ontwikkelings- en voortplantingsbiologie in het Animal Science Department van de University of Florida in Gainesville. In de periode tussen 1 april 1992 en 1 april 1996 werkte zij als assistent-in-opleiding bij de vakgroep Experimentele Diermorfologie en Celbiologie aan de Landbouwuniversiteit te Wageningen. De resultaten van het promotieonderzoek dat daar werd uitgevoerd zijn beschreven in dit proefschrift.

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