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GENETIC ANALYSIS OF GERM CELL MIGRATION DURING ZEBRAFISH EMBRYONIC DEVELOPMENT

Diplomarbeit

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von

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Erklärung

Die vorliegende Arbeit wurde im Labor von Dr. Erez Raz, Institut für Biologie I (Zoologie), Lehrstuhl für Entwicklungsbiologie, an der Albert-Ludwigs-Universität in Freiburg im Breisgau angefertigt.

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ABBREVIATIONS

A	adenin
ampR	ampicillin resistance
AP	alkaline phosphatase
APS	ammoniumpersulphate
β-lactamase / ßlact	penicillin amido-β-lactamhydrolase
Bmp	bone morphogenetic protein
bp	base pair
BSA	bovine serum albumin
BVDV	bovine viral diarrhea
CAT	chloramphenicol acetyl transferase
cDNA	copy DNA
C	cytosin
^{0} C	grad Celsius
CIAP	calf intestinal alkaline phosphatase
DMSO	dimethylsulfoxid
dNTP	2'-deoxinucleosid-5'triphosphate
E.coli	Escherichia coli
EDTA	ethylenediamin tetra-acetate
EMCV	encephalomyocarditis virus
ENU	ethylnitrosourea
et al.	et alii
flh	*floating head*
G	guanin
GFP	green fluorescent protein
h	hour
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
hpf	hours post fertilization
IRES	internal ribosomal entry site
LB	Luria-Bertani
M	molar
MCS	multiple cloning site

mM	millimolar
mRNA	messenger RNA
neoR	neomycin resistance
nm	nanometer
ntl	*no tail*
OD	optical density
ORF	open reading frame
pA	polyadenylation site
PCR	polymerase chain reaction
PGC	primordial germ cell
PI-G	phosphatidylinositol-glycan
PLAP	human placental alkaline phosphatase
PMG	posterior midgut
pmol	picomol
SA	splice acceptor
SEAP	secreted human placental alkaline phosphatase
spt	*spadetail*
T	thymin
T_A	annealing temperature
T_M	melting temperature
tris	tris(hydroxymethyl)aminomethan
UAS	upstream activating sequence
5’ UTR	5’ untranslated region
VEGF	vascular endothelial growth factor
v/v	volume per volume
w/v	weight per volume
YT	baco yeast ectract / baco tryptone

1. INTRODUCTION

1.1 The germ line

Two general mechanisms for germ cell formation are found throughout the animal kingdom: In some groups of organisms including insects, roundworms and vertebrates a clear segregation of the germline from the soma occurs early in embryonic development (e.g. celularization in *Drosophila* (Sonnenblick, 1950), day 7 post fertilization in the mouse (Ginsburg et al., 1990)) and marks the most basic dichotomous decision in development (Wei and Mahowald, 1994). In other species, like cnidarians, flatworms and tunicates the decision between the somatic and germ cell fate can take place even in adult animals so that the somatic cells do not lose their ability to become germ cells (Gilbert, 1997). Segregation of germ cells from the soma can be a result of unequal distribution of cytoplasmic determinants or a result of an induction process (McLaren, 1999). Pole cell and subsequently germ cell determination by localized cytoplasmatic determinants in *Drosophila* (Hay et al., 1988) or the P granules in *C. elegans* (Strome and Wood, 1983) are well known examples for this strategy. *Xenopus* embryos have structures microscopically similar to polar granules (reviewed in (Wylie, 1999)) and germ cell determination in zebrafish probably also results from early localization of germ cell determinants. In contrary, in mice and by extrapolation in all mammals, it seems that cells achieve germ cell status by induction and regulation (Lawson et al., 1999; McLaren, 1999). Evidence for inductive mechanisms for germ cell determination in mouse is given by transplantation experiments at day 6,5. Germ cells are normally located in the proximal region of the epiblast, but cell transplanted from distal regions to that place can contribute to germ line. Conversely, cells transplanted from the proximal regions to distal places do not form germ cells (Tam and Zhou, 1996). Interestingly, mice mutated for the *Bmp4* gene are deficient in germ cells, suggesting a direct or an indirect role for the *Bmp4* in germ cell induction (Lawson et al., 1999).

Interestingly, in most animals the germ cells are not formed in the gonads which is the region they are supposed to colonize. The primordial germ cells (PGCs) arise in other places and have to migrate through the developing embryo to find their appropriate location.

Once the germline lineage is specified, it is distinct from other lineages in its relative mitotic inertness during parts of development, and by its unique meiotic activities. Furthermore, in many but not all species, the germ cells have, when they appear in the embryo, the potential to differentiate into gametes of either sex, depending on signals from their environment (Wylie, 1999). It is impor-

tant to note that germ cells are totipotent and are capable of contributing to all cell lineages following fertilization (Wylie, 1999).

Despite the unique role and importance of germ cells, relatively little is known about the mechanisms through which they are formed and differentiate.

1.2 Germ cell migration in *Drosophila* and other animals – involved genes and tissues

In order to approach the unsolved questions of differentiation signals and migration factors for the germ cells of zebrafish, a comparison with other model organisms might be helpful. Considering the conservation of factors that regulate somatic development, it is likely that some aspects of germ cell development are shared not only on a morphological but also on the molecular level between *Drosophila* an other organisms, including zebrafish (Williamson and Lehmann, 1996). Therefore, knowledge of genes involved in *Drosophila* germ cell specification as well as knowledge of the interaction of germ cells with different tissues with which they interact during their migration can serve as a basis to plan experiments in the zebrafish system.

In *Drosophila* the PGCs, often referred to as pole cells, are the first cells to cellularise at the posterior pole of the embryo during mitotic cycle 10 (Sonnenblick, 1950). Cellularisation depends upon a specialized cytoplasm containing polar granules, which is maternally provided and assembles during oogenesis. Transplantation experiments proved that from the time of their formation, these cells are committed to the germ cell fate (Technau and Campos-Ortega, 1986; Underwood et al., 1980). At the blastoderm stage, the pole cells are located adjacent to prospective endodermal cells, which will give rise to the posterior midgut. During gastrulation these cells are carried along the dorsal surface of the embryo associated with the posterior midgut (PMG) primordium and are incorporated into the invaginating PMG pocket. Formation of the germ cells first outside the embryo proper, followed by migration through the posterior endoderm occurs in distantly related species such as *Drosophila*, mouse and *Xenopus* (Van Doren and Lehmann, 1997; Wylie, 1999).

At stage 10 of embryogenesis the pole cells migrate through the midgut epithelial layer. Coincident with this migration, the endodermal tissue in this region reorganizes by changes of cell-cell junctions and intercellular gap formation. At least in part, pole cell migration seems to require a rearrangement of endodermal cells (Jaglarz and Howard, 1995). After crossing the gut wall, the germ cells migrate along the basal surface of the gut to its most dorsal side. Then they move into the lateral trunk mesoderm, where segmentally repeated primordia that will give rise to fat body and somatic

gonad precursors resides. The directional migration from the endodermal to the mesodermal environment is achieved by a combination of repulsing and attracting signals. The germ cells are first directed to leave the entoderm by the *wunen* protein (Zhang et al., 1997) and then attracted towards the gonadal mesoderm by through the function of *columbus* protein, which is an HMG coenzyme A reductase (Van Doren et al., 1998).

In order to identify more genes involved in zygotic control of germ cell migration in *Drosophila*, a genetic screen was performed (Moore et al., 1998). Mutations affecting different stages of germ cell migration have been found. These efforts resulted in isolation of new alleles of known genes and isolation of new genes affecting each of the previously described steps of germ cell migration.

Mutations in *serpent* and *huckebein*, genes which affect gut development disrupt the ability of germ cells to pass through the gut wall. Mutations in *columbus, heartless* and *zinc finger homeodomain 1*, which act in mesoderm patterning, result in many germ cells remaining with the basal surface of the gut instead of moving into the lateral mesoderm. The function of the *fear-of-intimacy* gene is then required for the gonad coalescence. This gene seems to be involved in adhesive cell-cell interactions between the gonadal mesoderm cells. Most of the identified genes are expressed in the mesoderm which suggests that the environment provides a wide range of controlling factors.

Similar systematic genetic analysis and investigation of tissues which guide the pathway of migrating germ cells has not been performed in vertebrates.

1.3 Germ cell formation and migration in zebrafish

The Zebrafish offers several advantages over other model organisms for studying development. The fish are small and easy to breed, generation time is short and mature animals can lay hundreds of eggs every other week. The optical clarity of the externally fertilized eggs and the relatively fast development (summarized in (Kimmel et al., 1995)) enable easy examination of developmental processes. These properties allowed the performance of genetic screens for mutations affecting development.

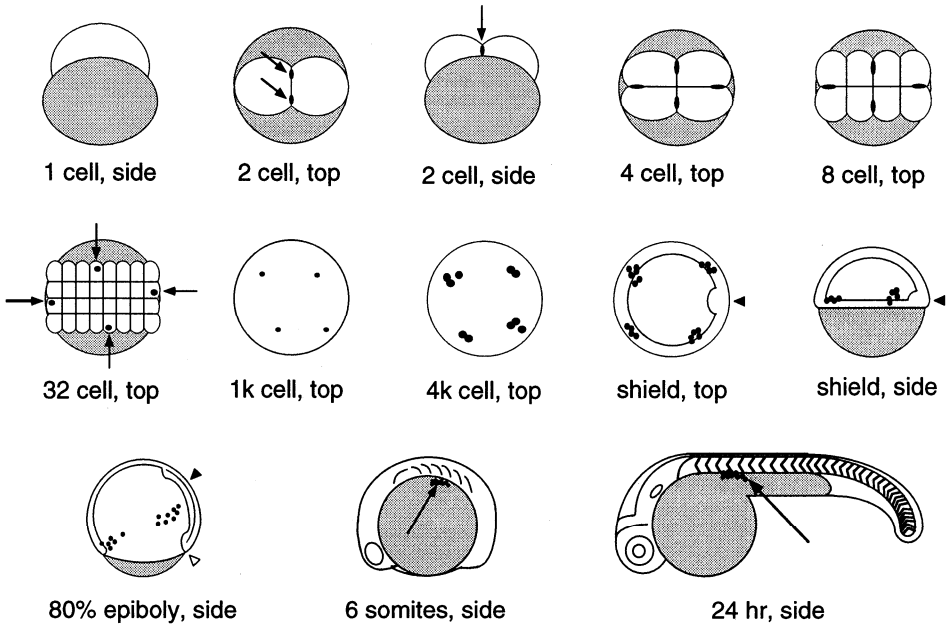
Although zebrafish is a popular model for studying vertebrate development, little is known about germ line development in this organism. Differentiation and migration of germ cells in zebrafish has so far been described using primarily morphological and histological criteria (Selman et al., 1993; van Winkoop et al., 1992). Therefore it is not surprising, that those descriptions focused on rather late stages when the germ cells have reached the ovary, which is formed by fusion of two paired primordia found on each side of the midline. In this place groups of germ cells are localized in nests surrounded by somatic “pre-follicle” cells. Subsequently, each cell is arrested in diplotene of the first

meiotic division and resides in a definitive follicle outside the nest. In the following stages, the oocyte grows in size, forms a vitaline membrane and accumulates yolk. Oocyte maturation ensues in which the first meiotic division is completed and the egg stops at the second meiotic metaphase. The mature eggs are then ovulated into the ovarian lumen capable of being fertilized. The second meiotic division is completed after the fertilization.

The analysis of those late stages of germ cell development cannot answer the questions about the origin of the cells contributing to the germ-line and their migration but at earlier stages they apparently have no distinct morphology and cannot be identified.

Two recent reports describe the cloning of the zebrafish *vasa* homologue (Olsen et al., 1997; Yoon et al., 1997). The *vasa* gene was initially identified in genetic screens in *Drosophila*, where it is required for germ cell development (Hay et al., 1988; Lasko and Ashburner, 1988). The spatial distribution of the *vasa* mRNA in zebrafish was determined by performing whole mount *in situ* hybridisations, allowing for the first time to follow the migration of germ cells from the place where they originated towards the gonad. The maternally provided *vasa* mRNA was detected first in 2-cell stage embryos where it is localized along the cleavage plane in short stripes of expression usually closer to the yolk (see figure 1). The same situation can be found in the 4-cell stage, when the RNA is also found along the second cleavage plane. The expression starts to condense into clumps as early as the 8-cell stage ending up in 4-cells at the 32-cell stage. Later, (at dome stage) the cells containing *vasa* have divided and 4-cell clusters can be detected, each containing 1 to 4-cells. At the shield stage, the 4 separate groups can be detected, spaced around the embryo and usually near the margin. Interestingly, the relation between the PGC clusters and the forming shield or dorsal side is random (Yoon et al., 1997). During epiboly until early somitogenesis, the PGCs migrate towards the dorsal aspect of the embryo, where they form two clusters of cells on either side of the notochord, at the level of the third to fifth somite. By 24 hours the cells are located at the anterior part of the yolk extension, extending posteriorly for a variable distance through early larval stages to form two bilateral rows of cells in the gonadal anlage.

Figure 1 (Yoon et al., 1997): Schematic summary of zebrafish germ line development during embryogenesis



Vasa expression as determined by whole mount *in situ* hybridization. The stages and views are indicated below each drawing and the yolk is shaded. Arrows point to *vasa* expression in selected stages. In shield stage embryos the black arrowhead indicates the dorsal shield and in 80% epiboly the developing notochord, whereas the white arrowhead indicates the germ ring. Further explanations are given in the text.

Using *vasa* mRNA as a marker, the germ cells of zebrafish can be localized and their migration can be monitored. The signals that lead to germ cell differentiation and the cues required for their proper migration in the zebrafish are currently unknown.

1.4 Investigation of zebrafish germ cell development

Investigating germ cell migration in zebrafish and finding the genes involved can be approached in several different ways. First, one could clone homologs for genes known to function in this process in other systems (see *Drosophila* examples above in section 1.2.). Examples for sequence and function conservation are found for instance for the *vasa* and *nanos*-like mRNAs, that are localized to the germ plasm in *Xenopus laevis*, (Forristall et al., 1995). *vasa* homologs have also been found in the germ line in mouse embryos (Fujiwara et al., 1994) and in zebrafish (Olsen et al., 1997; Yoon et al., 1997).

A second approach is based on the availability of fish lines carrying specific mutations affecting diverse developmental processes. These fish lines have been generated in large scale mutagenesis screens (Driever et al., 1996; Haffter et al., 1996). The offspring of fish heterozygous for a specific

mutation can be examined for altered germ cell development e.g. by performing in situ hybridization with *vasa* probes. As the identity of tissues located along the migration pathway of germ cells in wild type zebrafish is known, one could focus on mutations causing defects in these tissues.

Finally it is possible to identify new genes involved in germ cell specification and migration by additional genetic screens which focus on germ cells in a more direct way than the mutagenesis screens for visible phenotypes did so far. The examination of the offspring of mutagenized parents with *in situ* probes for *vasa* mRNA can help to circumvent the problem that early germ cells are morphologically indistinguishable from the surrounding tissue.

As this diploma work focuses on abnormal germ cell development in known mutations and on tests in order to build an efficient gene trap cassette for screens the last two items will be discussed in more detail.

1.5 Fish strains with mutations affecting the mesoderm development

As previously mentioned (Moore et al., 1998) the mesoderm provides important signals for germ cell migration in *Drosophila* which emphasizes the importance of this tissue. At shield stage in zebrafish the 4 groups of cells expressing *vasa* are spaced around the embryo and generally near the margin (Yoon et al., 1997) which is the region designated to become the mesoderm after gastrulation. (Kimmel et al., 1995; Kimmel et al., 1990). This is the reason why at least three known mutations for axial and paraxial mesoderm, *no tail (ntl), floating head (flh)* and *spadetail (spt)* are good candidates to be examined for altered germ cell migration.

no tail is the zebrafish homolog of the mouse *T (Brachyury)* gene (Schulte-Merker et al., 1994). *ntl* mutant embryos resemble mouse *T/T* mutant embryos in so far as they lack a differentiated notochord and the caudal region of their bodies, meaning the most posterior 11-13 of their normal 30 somites (Halpern et al., 1993). *ntl* is expressed transiently in all cells of the presumptive mesoderm, and later in the cells of the future notochord where it accumulates in the nuclei and is most probably needed to maintain its own transcription (Schulte-Merker et al., 1992; Schulte-Merker et al., 1994).

The zebrafish *floating head* (*flh*) gene encodes a homeodomain transcription factor (Talbot et al., 1995). Embryos lacking a functional *flh* gene product entirely lack notochord and have muscle tissue (fused somites) in the midline (Halpern et al., 1995). In contrast, *flh* mutants develop prechordal plate and other mesodermal derivatives, indicating that *flh* functions specifically in notochord specification (Talbot et al., 1995). Thus both *flh* and *ntl* mutations disrupt notochord development, but the *flh* and *ntl* phenotype are distinct: in the trunk of *ntl* mutants, mesenchymal cells proposed to be im-

properly or incompletely differentiated notochord occupy the midline, whereas in *flh* mutants the notochord precursors transfate into muscle cells (Talbot et al., 1995).

spadetail (*spt*) has been described as a mutation with defects in gastrulation movements resulting in a lack of trunk somites and a greatly enlarged tip of tail (Kimmel et al., 1989). *spadetail* mutants develop a relatively normal notochord (Griffin et al., 1998). These defects have been attributed to a reduction of convergent movements of ventrolateral cells towards the dorsal side (Ho and Kane, 1990). *In situ* hybridisiation using *myoD* as a probe shows, that the muscle precursors do not arrive at the appropriate places (Hammerschmidt et al., 1996). Instead, these cells remain in ventrolateral positions and eventually end up in the tail bud.

spt was found to encode a T-box transcription factor and is likely to be the key mediator of FGF signaling in trunk paraxial mesoderm (Griffin et al., 1998). Trunk and tail development are therefore dependent upon the complementary actions of two T-box genes, *spt* and *ntl*. (For more details see discussion).

1.6 Genetic screens

1.6.1 Chemical screens

Chemical genetic screens for identification of genes controlling developmental processes were first performed in *Drosophila* and in the *C. Elegans* systems. The first large scale chemical mutagenesis screens in vertebrates wereperformed in zebrafish using ethylnitrosourea (ENU), which induces point mutations (Mullins et al., 1994; Solnica-Krezel et al., 1994). The main advantages of ENU induced screens are their high efficiency compared to insertional mutagenesis and the fact that ENU induces point mutations instead of aberrations and deletions that are difficult to recover and to analyze (which is the case for X-ray mutagenesis). Furthermore, chemical mutagenes are relatively unbiased in the chromosomal sites in which they induce mutations thus making random mutations and saturation screens possible. The main disadvantage of ENU mutagenesis is that cloning the affected gene often proves to be difficult. Cloning of ENU induced mutations is done using the “candidate gene” approach (e.g. (Schulte-Merker et al., 1994)) or by positional cloning which is relatively difficult and expensive (Zhang et al., 1998).

1.6.2 Insertional mutagenesis screens

Insertional mutagenesis screens allowed the isolation of many genes important for the development of *Drosophila* as well as the identification of genes that function during mouse embryonic development (Bellen et al., 1989; Joyner, 1991; Joyner et al., 1992).

Gene trapping by insertional mutagenesis is based on DNA elements, which can randomly integrate into the genome, and in case it integrates into an open reading frame of a gene, it destroys its function. Interrupting the sequence of developmentally important genes, the inserted DNA element does not only cause a visible phenotype in homozygots, but also facilitates the cloning of the gene using the inserted sequence as a tag. The gene trap vector can include a splice acceptor, thereby keeping it functional even when it is integrated into an intron. Including a reporter gene, e. g. β-galacdosidase (lacZ) in the gene trap cassette can in case of integration into an open reading frame lead to synthesis of a fusion protein which includes the marker protein. thereby marking the tissue in which it is expressed. Gene traps have mainly been used in the mouse system using retroviruses as vectors (Joyner, 1991; Joyner et al., 1992).

Enhancer traps serve as a tool for detecting developmental genes based on their expression pattern rather than on their mutant phenotype. In such screens, performed mainly in *Drosophila*, expression of a reporter gene is regulated by nearby enhancers acting on a minimal promoter included in the transposon enhancer trap cassette. If the construct integrates near an enhancer, the transcription of the reporter gene driven by the minimal promoter can be visualized and examined. If the reporter does not integrate in a region controlled by an enhancer the promoter remains silent.

The P-element based enhancer detection in *Drosophila* allowed the identification of many genes functioning in specific developmental processes. For example, enhancer trap screens were performed in order to identify genes important for the function of the *Drosophila* immune system (Braun et al., 1997), or genes that function during *Drosophila* oogenesis (Grossniklaus et al., 1989). P-elemtent mediated screens are a very powerful tool in *Drosophila*, but can not be used in other sepcies due to requirement for host factors. Similarly, in the prime model organism of plant development, *Arabitopsis thalian*, an efficient, but plant specific transposable element of the Ac/Ds system from maize is available (Sundaresan et al., 1995). Insertional mutagenesis in the mouse is based on totipotent stem cells, which can be infected or transfected with a gene or enhancer trap construct in cell culture and then returned to the embryo, were they participate in the normal development of a chimeric mouse (Joyner, 1991; Joyner et al., 1992; Korn et al., 1992). Unlike in the model organisms mentioned above, insertional mutagenesis screens using gene trap or enhancer trap approaches cannot be performed in zebrafish at present which is mainly due to lack of proper vectors.

1.6.3 Transposon mediated insertional mutagenesis in zebrafish

For insertional screens in zebrafish, an efficient transformation vector, that is capable of expressing dominant markers would be needed. It was found that pseudotype retroviral vectors are able to infect zebrafish cells and that the integrated vector can be transmitted through the germ line (Burns et al., 1993; Lin et al., 1994) thus making insertional mutagenesis screens in zebrafish feasible, albeit at a much lower efficiency than that of the chemical mutagenesis screens (Gaiano et al., 1996; Gaiano et al., 1996). However, since as genes inserted within the proviral genome apparently cannot be expressed after germ line transmission in zebrafish, gene trapping and enhancer trapping are currently not possible with this system. Furthermore, insert-size limitations, deletions and rearrangement of the proviral sequences (Gaiano et al., 1996) may to some extent limit the usefulness of retroviral vectors for general transgenesis.

One option for insertional mutagenesis in fish is to use transposons (Fadool et al., 1998; Raz et al., 1997; Weinberg, 1998). The basic components of the transposon are a gene encoding the enzyme necessary for transposition, a transposase and flanking sequences required for recognition by the transposase. It has been shown that elements of the *Tc1/mariner* family, which are found in many species of the animal kingdom (Radice et al., 1994; Robertson, 1995) can function in zebrafish, because the transposition is independent of host specific factors (Fadool et al., 1998; Raz et al., 1997). This system could be used for gene trap and enhancer trap in zebrafish and elements which will be included into the trapping cassette should be tested.

An inherited problem in gene trap schemes is that only in one out of three cases the marker gene will be fused in-frame to the coding region of the trapped gene. This problem can be solved by using special sequences identified first in viruses. Internal ribosomal entry sites (IRES) allow ribosomes to bind mRNA not only from the 5’ cap as usual, but to start translation from this special IRES sequence (Mountford and Smith, 1995; Witherell et al., 1995) (more details see results). Therefore defining an IRES, which can function in zebrafish, and including it in front of the reporter gene in the gene trap cassette, would make the translation of the reporter independent of the integration frame.

1.7 Aims of this study

The goal of this study is to contribute to the investigation of the formation and migration of germ cells in zebrafish. As previously mentioned, different approaches can be taken. This diploma work focuses on abnormal germ cell development or migration in mutants with mesodermal defects on the one hand and on finding an IRES, which is functional in zebrafish on the other hand. Using both ap-

proaches, study of the development and morphology of zebrafish embryos and methods of molecular biology were combined.

The examination of different known mutants is done by analyzing embryos at different points of time using whole mount *in situ* hybridization with the *vasa* probe to label the germ cell. Mutants displaying defects in germ cell patterning could then be selected and examined at different stages performing *vasa* single stainings or double stainings with known markers. The results of these experiments will shed light on the identity of the tissues involved in the migration and guidance of the germ cells at different stages of development.

Three different IRES sequences from viruses and mouse have been examined for their activity in zebrafish. This phase of the work included the cloning of these sequences between two reporter genes, establishing assays in order to monitor the activity of these reporters, and the testing of the different constructs themselves by injections. The results obtained in this part will allow one to decide whether an IRES, and if so which IRES, can be inserted into a gene and enhancer trap cassette for transposon mediated insertional mutagenesis.

2. MATERIAL

2.1 Chemicals and buffers

All chemicals used for the preparation of the different solutions listed below were obtained from Gibco BRL, Berlin; Merck KG, Darmstadt; Carl Roth GmbH, Karlsruhe or Sigma-Aldrich, Deisenhofen in the quality “pro analysis”. The recipes for the different buffers are listed in the appropriate place in the method part.

2.2 Enzymes

2.2.1 Restriction enzymes (with appropriate buffers)

Cla I, EcoR I, EcoR V	Boehringer Mannheim
BamH I, Kpn I, Not I, Sac I, Sty I	Gibco BRL, Berlin
Afl II, Sal I, Sma I, Spe I, Xba I	MBI Fermentas, Littauen
Xho I, Xmn	New England Biolabs, USA

2.2.2 Other enzymes (with appropriate buffers)

Phosphatase, alkaline from calf intestine 1U/µl	Boehringer Mannheim
Penicillinase from Escherichia 100-300 units per mg using benzylpenicillin 20-40 units per mg solid using chephaloridin	Sigma, Deisenhofen
DNAse I, RNAse-free	Boehringer, Mannheim
Pronase	Sigma, Deisenhofen
Proteinase K	Boehringer, Mannheim
SP6 RNA - Polymerase	MBI Fermentas, Littauen
T4 DNA ligase	New England Biolabs, USA

2.3 Kits

Isolation of DNA from agarose gels:	
GeneClean II Kit	Bio 101, USA
PCR:	
Advantage - HF™ PCR kit	CLONTECH, USA
Plasmid-DNA purification:	
QIAfilter Midi Plasmid Kit	Qiagen, Hilden
QIAfilter Maxi Plasmid Kit	Qiagen, Hilden
QIAprep Spin Miniprep Kit	Qiagen, Hilden
Sequencing:	
Thermo Sequenase Cycle Sequencing Kit	Amersham, Braunschweig
Sequi Therm Excel™ II Long-Read™ DNA Sequencing Kit LC	Epicentre Technologies, USA
mRNA-transcription:	
SP6 mMESSAGE mMACHINE™ Kit	Ambion, USA

2.4 Bacteria and media

Bacteria strains:	
E.coli TOP10F'	Invitrogen, USA
E.coli DH5α	Gibco BRL, Berlin
Media:	
2xYT-medium	16g trypton, 10g yeast extract and 5g NaCl in 1 liter ddH_2O
LB-medium	10g trypton, 5g yeast extract, 10g NaCl in 1 liter ddH_2O, adjusted to pH 7,5 with NaOH

SOC	20g trypton, 5g yeast extract and 0,5g NaCl, 10 mM $MgCl_2$, 20 mM glucose in 1 liter ddH_2O

To pour plates 1,5% Agar (w/v) was added. Selective media and plates were produced by adding 60-80 mg/l ampicilline. As ampicilline is not heatstable it was added after autoclaving and plates were poured at 40 -50^0 C.

Bacteria are stored by adding 25-30% glycerol to 1 ml of a liquid culture and freezing at -20^0 C for temporary and -80^0 C for long term storage.

2.5 Used DNA

2.5.1 Oligodeoxyribonucleotides

All oligonucleotides were produced by MWG Biotech in Ebersberg, Germany.

Oligodeoxyribonucleotides for PCR

homologous regions are printed in bold letters

	PCR-Produkt	*PCR size*
upstream primer	Sequence 5' ---> 3'	T_M (^{0}C)
downstream primer	Sequence 5' ---> 3'	T_M (^{0}C)
	PLAP without signal sequence 1.ORF	*1581 bp*
980901 AP1 up	CCATCGAT TATATC ATG**GGGCATCATCCTAGTTGAG** Cla I mut. Eco RV start	68,3
980901 AP1 down	AGAATTCTACTAGTTTCTAT**TCA**GGGAGCAGTGGCCGTC Eco R I stop Spe I stop	71,6
	β-lactamase without signal sequence 1.ORF	*832 bp*
980901 βlact1 up	CCATCGAT TATATC ATGGGG**CACCCAGAAACGCTGGTG** Cla I mut. Eco RV start	73,8
980901 βlact1 down	AGAATTCTACTAGTTTCTAT**TTA**CCAATGCTTAATCAG Eco R I Spe I stop	64,1

	PLAP without signal sequence 2.ORF	*1569 bp*
980901 AP2 up	TTGAATTC GATATC ATG**GGGCATCATCCTAGTTGAG** Eco R I Eco RV start	67,1
980901 AP2 down	TTCTCGAG **TCA**GGGAGCAGTGGCCGTC Xho I stop	71,0
	β-lactamase without signal sequence 2.ORF	*818 bp*
980901 βlact2 up	TTGAATTC GATATC ATGGGG**CACCCAGAAACGCTGGTG** Eco R I Eco RV start	72,7
980901 βlact2 down	TTCTCGA **TAC**CAATGCTTAATCAG Xho I stop	60,1
	BVDV IRES	*385 bp*
BVDV-IRESup	**GTATACGAGAATTAGAAAAGGCAC**	57,6
BVDV-IRESdown	**G**ATATC**TGTACAGCAGAGATTTTTAG** Eco R V	58,5
	EMCV IRES	*645 bp*
980615SA5'Spe	CCCACTAGT**TTGTATTGTTACAGCATTG** Spe	60,7
980525IRES3'	AAGA**TATCA**TCGTGTTTTTCAAAG Eco R V	54,2
	VEGF IRES	*162 bp*
Israel-IRES-up	AGCGCAGAGGCTTGGGGCAGCCGAGCGGCAG**CCAGGCC** **CCGGCCCGGGCCTCGG**	> 75,0
IRES-ECORVrev	**G**ATA**TC**GGAGGCCGTCCGGGGCCG Eco R V	73,0
	PLAP with signal sequence	*1627 bp*
981203PLAPfull5'	AAAATCGATATC **ATG**CTGCTGCTGCTGCTGCTGCTGGGCCTGA Cla I Eco R V start **GGCTA**	> 75,0
981203PLAPfull3'	TTTTCTAGACTAGTTTCTATT**TCA**GGGAGCAGTGGCCGTCT Xba I Spe I stop stop	71,5
	SEAP	*1540 bp*
981203SEAP3'	TTTTCTAGACTAGTTTCTATT**TCA**CCCCGGGTGCGCGGCG TCGGT **Xba I Spe I stop stop**	> 75,0

Special oligodeoxyribonucleotides for sequencing

Name	*Function* Sequence 5' ---> 3'	T_M (^{0}C)
980930AP 668	*internal priming in the PLAP at position 668 (sequencing 5' ---> 3')* CAGCTCATCTCCAACATGGAC	59,8
101398AP 940 rev	*internal priming in the PLAPat position 940 (sequencing 3' ---> 5')* GTGTGGAGTCTCGGTGGAT	58,8
AP Seq 3'	*priming at the 3' of the first PLAP ORF (sequencing 5' ---> 3')* CCGCGTTGCTTCCTCTGC	60,5
βlact Seq SpeI	*priming at the 3' of the first βlact ORF (sequencing 5' ---> 3')* GCATTGGTAAATAGAAAC	46,9

Standard oligodeoxyribonucleotides for sequencing

Name	Sequence 5' ---> 3'	T_M (^{0}C)
SP6	ATTTAGGTGACACTATAGAATAC	53,5
T7CS2+seq	GTAATACGACTCACTATAG	50,2
M13 (forward)	GTAAAACGACGGCCGT	50,0

2.5.2. Used plasmids

BLP-plasmid — Plasmid containing the T7 promoter, the BVDV IRES and a luciferase gene downstream of the IRES, obtained from Raul Andino (University of California, San Francisco).
Used to amlify the BVDV IRES.

CLA 12 AP: — Containing human PLAP between polylinkers and including the amp^r gene.
Used to amplify different forms of alkaline phosphatase.

PBluescript SK — Containing a f1 origin, lacZ gene, multiple cloning site and the amp^r gene.
Used to amplify β-lactamase.

pBS-Silen: pBluescript KS containing the splice acceptor of the zebrafish *vasa* intron number 6; EMCV IRES (with 11th ATG changed to HindIII site), LacZ, *Xenopus* EF1A enhancer/promotor, neo^r gene, SV40 polyadenylation signal (constructed by Gilbert Weidinger, personal comunication).
Used to amplify SA / EMCV IRES.

PBS-zfVLG-Bgl/Eco pBS containing $^1/_3$from the 3' end of the zebrafish *vasa*-like gene subcloned as a Bgl II/Eco RI fragment into BamHI/Eco RI sites.
Used to produce vasa antisense RNA for *in situ* hybridisation.

pCRII-TOPO: TA cloning vector with SP6 promoter and reverse T7 promoter within the LacZ gene, PCR insert is ligated to 3' T overhangs within the MCS. The pCRII-TOPO vector is covalently bound to topoisomerase I enzyme for fast and very efficient cloning.
Used to clone different PCR products.

pCR 2.1 TA cloning vector with the same features as pCRII TOPO but with an M13 priming site instead of the SP6 promoter. As the vector is not linked to topoisomerase, PCR products are inserted by T4 ligase.
Used to clone SA / EMCV IRES.

pCS2+ : Expression vector, containig a strong enhancer/promoter (simian CMV IE94), followed by a polylinker and the SV40 late polyadenylation site, SP6 promotor is present in the 5' UTR of the mRNA of the CMV promotor, T7 promotor in reverse orientation, the vector backbone is from pBluescript II KS+ and includes the amp^r gene.
Used to construct the 6 expression plasmids with the different IRESs.

pG1 Plasmid for testing promoter constructs using GFP as a reporter, containing mGFP5 chromophore, SV40 polyadenylation site, upstream and downstream polylinkers.
Used to clone GFP.

2.5.3 DNA size markers

Three different markers were used to monitor the size of PCR products and restriction fragments.

λ BstEII (λ DNA digested with BstEII)			New England Biolabs, USA			
Fragments:	8454 bp	7242 bp	6369 bp	5686 bp	4822 bp	4324 bp
	3675 bp	2323 bp	1929 bp	1371 bp	1264 bp	702 bp
	224 bp	117 bp				

1 kb marker			MBI Fermentas, Littauen			
Fragments:	10000 bp	8000 bp	6000 bp	5000 bp	4000 bp	3500 bp
	3000 bp	2500 bp	2000 bp	1500 bp	1000 bp	750 bp
	500 bp	250 bp				

100 bp marker			MBI Fermentas, Littauen			
Fragments:	1000 bp	900 bp	800 bp	700 bp	600 bp	500 bp
	400 bp	300 bp	200 bp	100 bp	80 bp	

2.6 Fish - Strains

All experiments were performed with zebrafish (*Danio rerio*, synonym *Brachyodanio rerio*). For injections the wild type strains AB, TL and AB/TL were used. Fish heterozygot for *spt*, *flh*, and *ntl* were in an AB or TL-background. The *spt* allele used was b 104, the *flh* allele was ak 41a, and the *ntl* one b160.

2.7 Technical equipment

In this section only equipment, which was specially needed to carry out this diploma work, is listed. Equipment that is used daily in molecular biology are not listed.

Injector	Eppendorf Microinjector 5242
Needle Puller	Kopf vertical pipet puller model 720
Microscope	Zeiss Axioplan 2
Camera	ProgRes 3008 (Kontron Elektronik)
Sequencing machine	DNA-Sequencer Long Reader 4200 (Li-COR)
Absorbance measurement	Microplate reader model 550 (Bio Rad)

2.8 Software

Reading of sequencing gels	Base ImagIR
Sequence analysis	MacVector 6.1, Sequencer 2.8
Map drawing	Mac Plasmap 2.1
Image processing	Adobe Photoshop 5.0
Word processing	Microsoft Word 98
Data analysis	Microsoft Excel 98

3. METHODS

3.1 PCR

The polymerase chain reaction allows to amplify smallest amounts of DNA exponential if the 3' and the 5' end of the sequence are known and the sequence is not too long. During the procedure the doublestranded template DNA is melted (denatured) and synthetic oligonucleotides which fit to the known 3' and 5' ends are bound to the singel strands (annealed). The last step is the elongation of the short primers by a heatstable polymerase. The annealing temperature depends on the length and the bases of the used primers and the elongation time varies with the length of the DNA template (Saiki et al., 1988). These steps are repeated up to 30 times. As the DNA produced in each cycle serves as a template for the next cycle, the total amount of DNA increases exponentially.

3.1.1 Reaction mix

The typical PCR reaction was carried out in a total volume of 25 µl containing 50-150 ng template DNA, 2,5 µl 10x PCR-Buffer, 2,5 µl 10x dNTP mix, 50 pmol of each primer and 0,5 µl 50x polymerase mix.

To avoid interaction between the template and primer sequences and the 3'-5' exonuclease activity of some of the polymerases, which normally serves for correction of wrongly integrated bases but could also lead to degradation of single strand DNA, the enzyme was always put as the last component into the mix. Unsuccessful reactions were repeated with changed concentrations of template DNA or primers.

The reaction mix was always covered with a drop of mineral oil to prevent evaporation. All the reactions were done with the Advantage - HF^{TM} PCR kit from CLONTECH, USA.

3.1.2 Amplification

The different steps of the PCR require different temperatures and duration, which are entered into the memory of the PCR machine before starting the procedure. The following data are not absolute values but parameters inbetween which the reactions were done.

Step 1	95^0 C	2-4'	denaturation

The double strands separate completely into single strands for the first time, longer templates or templates with a high amount of G and C need a longer denaturation time.

Step 2 95^0 C 20-30''denaturation

The template and the newly synthesized strand melt and split after each round of annealing and elongation.

Step 3 45-72^0 C 20-30''annealing

During this step the primers bind to the complementary sequence of the template DNA. In order to get specific instead of unspecific interaction the annealing temperature should be over 45^0 C. The temperature used to bind the primers is typically 3^0 C below the theoretical melting temperature which can be calculated by the formula of Thein and Wallac:

$T_M = 2^0 C \times (n_A+n_T) + 4^0 C \times (n_C+n_G)$

with: n_A: number of adenin residues n_T: number of thymin residues

n_C: number of cytosin residues n_G: number of guanin residues

As there are always two primers in each reaction, one which primes 3' and one which primes 5' the lower of both possible temperatures is used. In most PCR-reactions the primers consisted of a region which bound to the template and a second part, which could not bind during the first cycle but contained restriction sites. As those restriction sites are amplified in the elongation step, too, the template changes and the annealing temperature could be increased after some cycles in order to have more specific priming.

Step 4 68-72^0 C 1,5-3,5' elongation

The bound primers serve as a starting point for the polymerase. The elongation time depends on the length of the template.

Step 5 x times back to Step 2

The number of cycles which are carried out is a compromise between the wish to produce a sufficient amount of DNA and the wish to avoid PCR mistakes occurring by wrong base-integration. While the first would lead to a high amount of cycles the second reduces the number of cycles. Usually 20 to 30 cycles produce a sufficient amount of DNA.

Step 6 68-72^0 C 4' elongation

This last elongation step is optional and can be introduced to make sure that no half elongated products are left.

Step 7 4^0 C

To suppress further reactions the mix is immediately cooled down.

The success of the reaction was checked on agarose gel. The DNA band with the expected size could be distinguished from the template and the primer bands and isolated from the gel.

3.2 Cloning of DNA fragments

In order to receive a DNA fragment in high copy numbers, e.g. a PCR product or a gene casette consisting of several DNA pieces, the fragment or cassette is inserted into the proper site of a plasmid or vector. During the process of transformation specially prepared bacteria strains are able to take those plasmids up. In the used strains the plasmid DNA is replicated independently of the bacterial chromosome, producing high copy numbers of plasmids.

3.2.1 Ligation

T4 DNA ligase catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA. The enzyme joins blunt end and cohesive end termini and is therefore suitable to link covalently a DNA fragment into plasmids and vectors. The molar amount of insert DNA fragments should be two or three times higher than the amount of open plasmid molecules. In a typical ligase reaction not more than 100 ng of total DNA (insert and vector) are incubated in a total volume of 10 µl containing 1 µl (1U/µl) of T4 DNA ligase and 1 µl 10x ligase buffer. The minimal incubation time at 14^0 C was four hours, most ligations were carried out overnight. If possible, two control ligations, one only with the insert and one only with the vector were added to check the frequence of religation.

3.2.2 Transformation into competent cells

Competent cells are bacteria, which are able to take up plasmid DNA by heat shock or electroporation. The bacteria, which are stored at -80^0 C are slowly thawed on ice. Normally 3 µl of the ligation, in cases with very little amounts of DNA up to 10 µl, were added to 50 µl of bacteria, stirred carefully and incubated 30 minutes on ice. The bacteria were forced to take up the plasmid by putting them at 42^0 C for 30 seconds and then again on ice for 3 to 5 minutes. Including the plasmid which carries the resistance gene against a special antibiotic, the cells achieve the possibility to survive in an environment containing this material.

Before plating the bacteria on selective plates containing ampicilline, they are grown for 30 minutes in 500 µl SOC. Usually not more than 150 µl were plated out using glass beads. Only colonies with the resistance gene should be able to grow overnight in a 37^0 C incubator.

3.2.3 Topo cloning

Topo cloning provides a highly efficient, quick single-step cloning strategy for direct insertion of Taq polymerase amplified PCR products into a plasmid vector. Taq polymerase has a nontemplate dependend terminal transferase activity which adds a single deoxyadenosin to the 3' ends of the PCR products. The linearised topo vector has single overhanging 3' deoxythymidin residues. This allows PCR inserts to ligate efficiently with the vector. Topo cloning was mainly used for quick sequencing of PCR-products.

3.2.4 Testing bacterial clones for plasmid integration

Depending on the background of the control ligation, 4 to 30 single clones per plate were picked and raised over night in 2 ml of LB or 2xYT media on an 37^0 C shaking incubator. The plasmid DNA was isolated with QIAprep Spin Miniprep Kit according to the Quiagen standard protocol. In order to test whether the plasmid contains the desired insert or not a double restriction digest (as described previously) was performed using enzymes which cut the whole insert or part of it out of the vector. Whether DNA pieces of the expected size fell out or not was detected by agarose gel electrophoresis.

Sufficient amounts of clean plasmid DNA were received using larger culture volumes and QIAfilter Midi or Maxi Plasmid Kit.

3.2.5 Gel electrophoresis and checking DNA

Electrophoresis was carried out in 0,5-2,0% (w/v) agarose (obtained from GIBCO, BRL) in TAE buffer. To monitor the DNA or RNA ethidiumbromid (0,5µg/ml) was added. Typically 0,1 - 1µg DNA or RNA mixed with loading buffer were loaded in each lane and 5-10 V/cm were applied to run the samples into the gel. To monitor RNA special denaturing buffer (Ambion) was used and the samples were heated at 65^0 C for 2 minutes. If electrophoresis was used to separate different restriction fragments or PCR products, the product with the expected size was cut out with razor blades under UV-light. Gel electrophoresis was also used to calculate the concentration of DNA or RNA samples in comparison to markers with known concentration.

To check the purity of DNA or RNA solutions usually 1µg was diluted in 99µg H_20 and measured in a photometer at 260 nm and 280 nm. The ratio 260 nm/280nm should be around 1.8 for DNA and slightly higher for RNA.

TAE-buffer (1x)

40 mM tris-acetat 1 mM EDTA (pH 8,0)

Gel loading buffer (6x)

0,25% (w/v) bromphenolblue	0,25% (w/v) xylencyanol	30% (v/v) glycerin

3.3 Sequencing of DNA

The sequencing was done according to the method of Sanger (Sanger et al., 1977) with 4 different PCR reactions containig the normal bases and a small amount of one dideoxynucleotide, which terminates the elongation. The DNA fragments of different sizes were separated by using polyacrylamid gels.

3.3.1 Pouring of polyacrylamid gels

The gel was poured between two glass plates, previously cleaned with water and ethanol and assembled with 25µm plastic spacers. For this the chemicals were mixed and filtered sterile. After pouring the gel a spacer was added at the top. After solidifying for one hour this spacer was replaced by the loading comb.

Gel:

25,5 ml ddH_20	10 ml 5 x TBE	21 g ureter
50 µl TEMED	500 µl DMSO	350 µl APS (10%)
5,8 ml Rapid Gel-XL concentrat		

TBE (5x)

450 mM tris	5 mM EDTA	450 mM bor acid

3.3.2 Reaction

In a typical sequencing PCR reaction 200 ng of DNA and 2 pmol fluorescent labeled primer were filled up with ddH_20 to a total volume of 13 µl and split in 4 reactions. 4 different mixes consisting of the nucleotides, one dideoxynucleotide and a DNA polymerase without 3’ ---> 5’ exonuclease activity were then added to the reactions. For as long as possible the fluorescent primers were kept in darkness.

If the sequencing was not satisfying, the template DNA was previously cleaned with phenol chloroform, the amount of template or primers was changed or different PCR kits were used.

The reactions were stopped by 3 µl stop-buffer and denatured 10 minutes at 70^0 C before loading in the gel slots in order to obtain single strand DNA fragments.

3.3.3 Gelelectrophoresis and analyzation

The DNA fragments of different size were separated with the Sequencer 4000L (Li-cor) using the following settings for a 66 cm gel:

Voltage: 2000 V	Current: 25mA	Power: 45 W
Heater: 45^0 C	Frames: 35	Signal gain / offset: 440 / 160

The vertical hanging gel was assembled with two buffer chambers at the bottom and the top containing 1x TBE and preheated before loading the samples. Focus and background were automatically corrected by using the Li-cor software. Due to their size small DNA fragments passed the scanner region at the bottom of the sequencer first and were detected by the fluorescent primers using laser technics. The scanned pictures were stored on hard disc and translated automatically into bases by Bas ImagerIR software. If only a few ambiguities occurred they were corrected manually. Regions with too many ambiguities were excluded. All data was assembled on the computer using the software Sequencer 2.8.

3.4 mRNA and DNA injection

3.4.1 Technical preparations

Transcription of mRNA

Usually 5µg plasmide DNA containing the insert, which should be transcribed into mRNA was cut with an appropriate enzyme downstream of the insert and afterwards cleaned with phenol chloroform precipitation. Only 0,5 µg of DNA were inserted into the reaction by cutting the standard message machine protocol down to 50%. After two hours of transcription for the SP6 promotor the reaction was stopped by digesting the template with RNAse free DNAse. The mRNA concentration was determined by running 0,5 µl on an agarose gel. Afterwards the mRNA solution was aliquoded and stored at -80^0 C.

Preparation of DNA

The DNA constructs were injected as circular plasmides using pCS2+ as a vector. The DNA was not specially cleaned after the Quiagen midi or maxi preparation and diluted in 10 mM HEPES to a concentration of 20 ng/µl or lower.

Equipment for injection

In order to protect the dechorionated embryos all petridishes were covered with 2% (w/v) agarose in 0,3 x Danieaus. For the injection procedure special dishes with ramps were prepared by inserting a coverslip sloping into the liquid hot agarose and removing it after solidification. All dishes were covered with 0,3x Danieaus.

To produce needles, glass capillaries of 1 mm in diameter were inserted into a micropipet puller and pulled with the following parameters: heat 575, pull 170, velocity 50 and time 150. Subsequently the needles were cut with a razor blade or broken with forceps to open them.

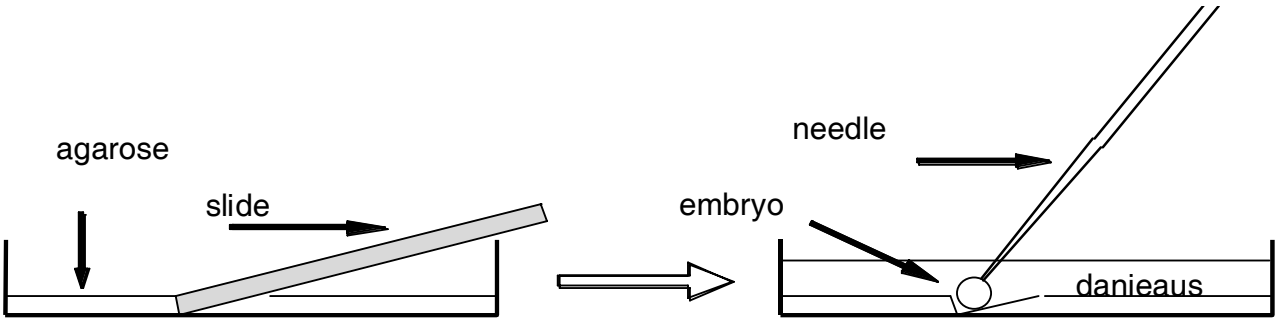
Danieaus (30x stock solution):

174,0 mM NaCl	21,0 mM KCl	12,0 mM $MgSO_4$
18,0 mM $Ca(NO_3)_2$	ph 7,6	

3.4.2 Dechorionisation

After *in vivo* or *in vitro* fertilization embryos could be dechorinated by incubation in 4 mg pronase dissolved in 10 ml 0,3x danieaus. The first embryos hatch usually after 2 to 3 minutes. Washing them extensively with 0,3x Danieaus most of the embryos leave the chorion and are ready for injection.

Figure 2: Preparation of ramps and injection of embryos



3.4.3 Injection

Injection needles were loaded using special microloader tips from Eppendorf. After inserting the needle into the fixture of the injector, the amount of injected liquid could be regulated by injection time, injection pressure and backpressure. Most injections were done using constant high backpressure and visual control of the amount of injected liquid. Embryos were injected with mRNA up to 4-cell stage, DNA injections were not done after 2 cell stage.

After injection all embryos were transfered into new dishes and raised in 28^0 C incubators. Dying embryos were removed every hour. In order to observe the starting point of transcriptional activity some embryos were injected with GFP and monitored under specially filtered light.

3.5 Protein extraction and colorimetric assays

3.5.1 Protein extraction

Injected embryos were raised and assayed for translation of alkaline phosphatase and β-lactamase at different times. In order to avoid too many undesirable proteins the yolk was chopped off with forceps or the embryos were heated to 65^0 C. Yolk protein fell out and the solution was clarified by centrifugation. Usually about 10 embryos, which had previously injected with the same construct, were smashed in 1,5 µl of extraction buffer per embryo using 1,5 ml Eppendorf tubes and plastic pistils.

Several extraction buffers especially with different concentrations of tris and detergents were tested for their ability to extract proteins and their influence on the colorimetric assays. The buffer finally used is considered to serve both goals: A extraction of thePLAPand the β-lactamase protein and no harmful effects on the assays. A protease inhibitor (Comlete^{TM} Mini, Boehringer Mannheim) was added and the embryo lysate was always put on ice in order to avoid protein degradation during the following steps.

3.5.2 Total protein assay

The total protein assay (Protein Assay, Bio-Rad, München) was conducted as described in the standart protocol and served to determine the total amount of proteins in a given solution (Bradford, 1976). The absorbance shift of Coomassie Brilliant Blue G-250 from 465 nm to 595 nm when binding to protein occurs and the subsequent change in optical density was measured at 595 nm with a Microplate Reader Model 550 from Bio-Rad.

Generating a standart curve with known concentrations of BSA allowed to calculate the protein concentrations in different embryo lysates and to dilute all of them to the same concentration before performingPLAPand β-lactamase assays.

3.5.3 Alkaline phosphatase and β-lactamase assays

The evaluation of these assays is described in the result section.

3.6 Whole mount *in situ* hybridization

All whole mount *in situ* hybridizations were done according to the standart protocol from C. and B. Thisse.

3.7 Breeding fish, handling of embryos

Crossing of fish, harvesting and sorting eggs as well as the incubation of embryos and raising in or outcrosses were done as described in “The zebrafish book. A guide for laboratory use of zebrafish (*Brachyodanio rerio*)” (Westerfield, 1994).

In order to take pictures embryos were embedded in 100% glycerol.

4. RESULTS

The results sections will be split into two parts one concerning the analysis of germ cell migration in mutants with defective mesoderm differentiation (section 4.1) and the a second part describing the evaluation of IRES elements in zebrafish (section 4.2).

4.1 Germ cell migration in zebrafish mesoderm mutants

4.1.1 Phenotypes of *no tail*, *floating head* and *spadetail* at 24 h hpf

The germ cells in zebrafish appear to segregate from the soma as early as the 32-cell stage and then have to migrate from their original position in order to reach their final location (see introduction). Signals provided by different tissues along the migration path as well as signals provided by the target tissues are likely to serve as directional cues for the germ cells.

At 24 hours of development the germ cells are normally found on either side of the axis at the anteroposterior level of the third to the fifth somite. To check if axial mesoderm or paraxial mesoderm are important for the normal migration pattern determined the position of the germ cells at 24 hours in mutants affected in development of mesodermal derivatives was determined. An additional criteria to look at in *in situ* hybridized mutant embryos is the altered numbers of germ cell compared to the wild type siblings.

Fish heterozygous for the mutations for either *no-tail* (*ntl*), *floating head* (*flh*) or *spadetail* (*spt*) were crossed and the resulting progeny was fixed at 24 hpf. Subsequently *in situ* hybridization was performed using *vasa* as a germ line specific marker.

ntl and *flh* - phenotype

In comparison to wild type siblings in both, *ntl* and *flh* mutant embryos, no altered germ cell migration pattern was found. As shown in Figure 3 (lateral view) the germ cells arrive at the proper place near the anterior yolk extension and form clusters. The dorsal view (Figure 4) reveals that two clusters are formed on either side. Thus, the axial mesoderm is neither essential for attracting the germ cells to the proper position nor for the spacing of the two clusters.

In addition, the cluster sizes of the *ntl* mutants and the *flh* mutants were compared to that of the wild type siblings. Overall no changes in cluster size or cell number were observed.

vasa expression at 24 h in different mutants

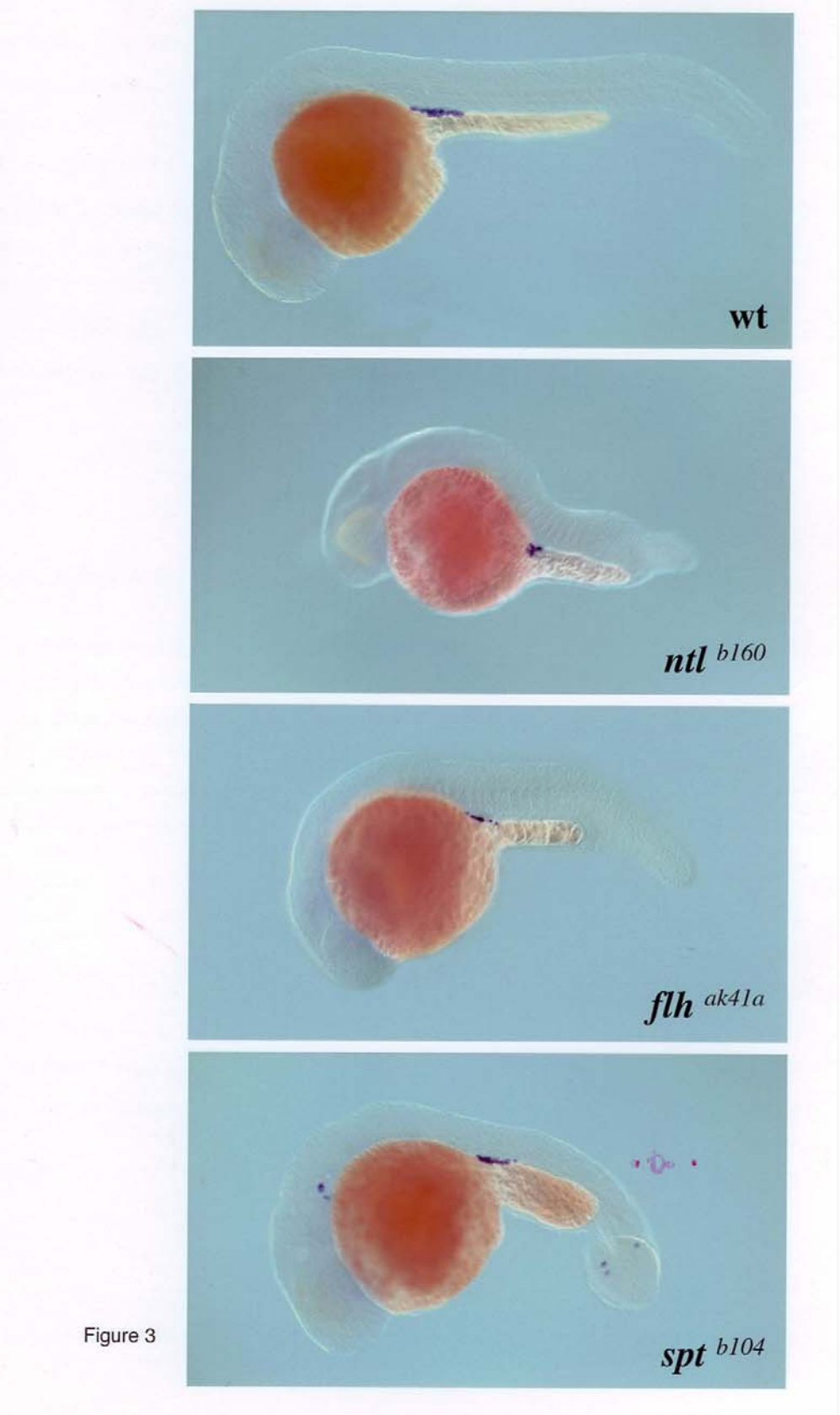


Figure 3

vasa expression at 24 h in different mutants

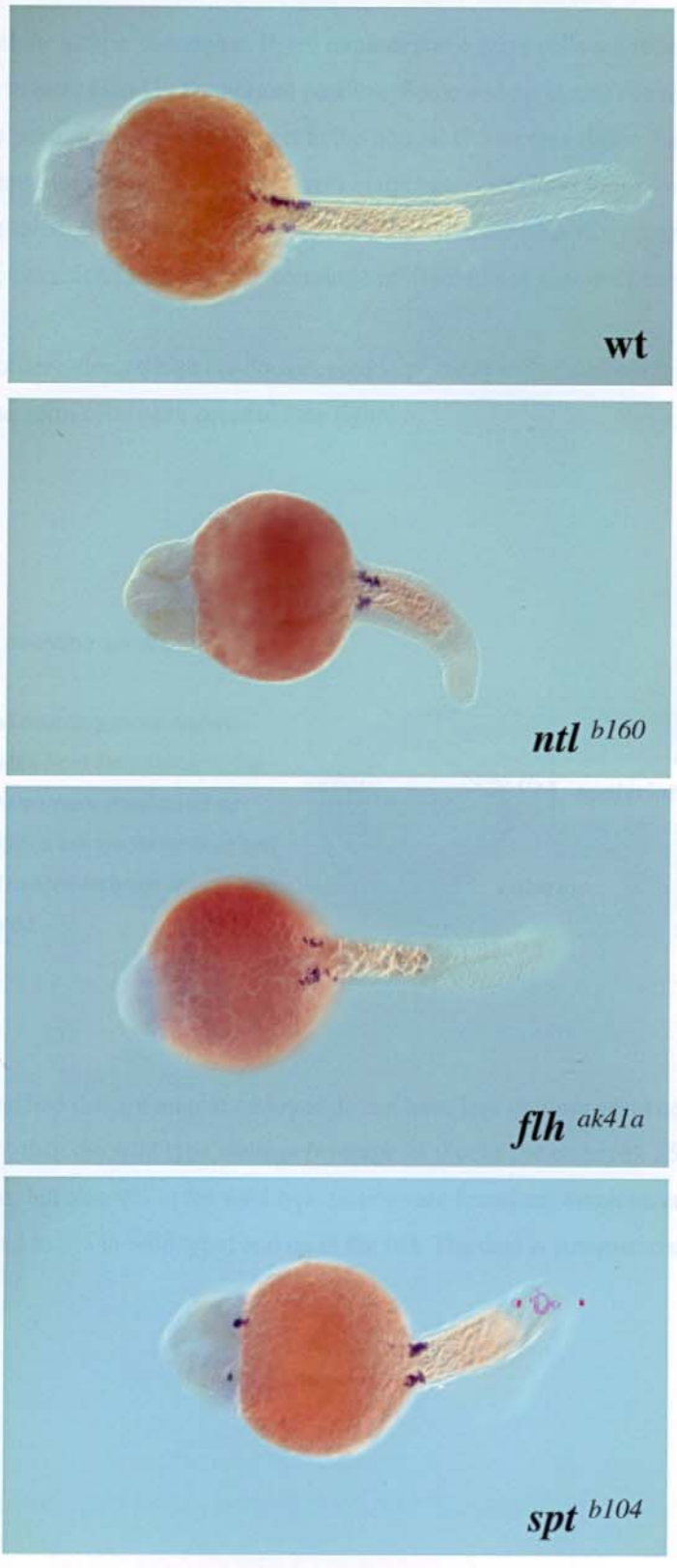


Figure 4

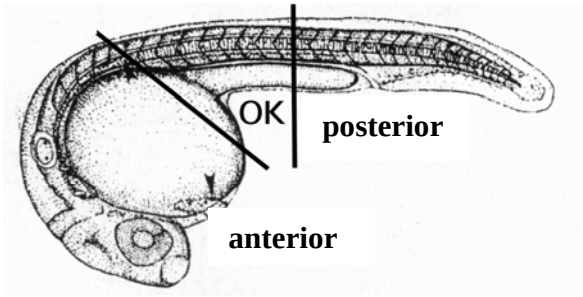
spt - phenotype

spt mutant embryos show a clear phenotype. In *spt* mutants some germ cells are found in two ectopic locations in addition to cells found in the normal position. Some end up in anterior regions and some can be found in more posterior regions in respect to the normal cluster (see figure 3 and figure 4). While nearly all the anterior ectopic germ cells arrive at the same anterioposterior level half way between the midbrain-hindbrain boundary in the mutant embryos, the posterior ectopic cells are spread from the yolk extension to the "spade"-forming undifferentiated mesenchyme at the tip of the tail.

To support these observations which are documented in pictures and to address this problem in a more detailed way the germ cells were counted (see figure 5).

Figure 5: Regions for counting germ cells

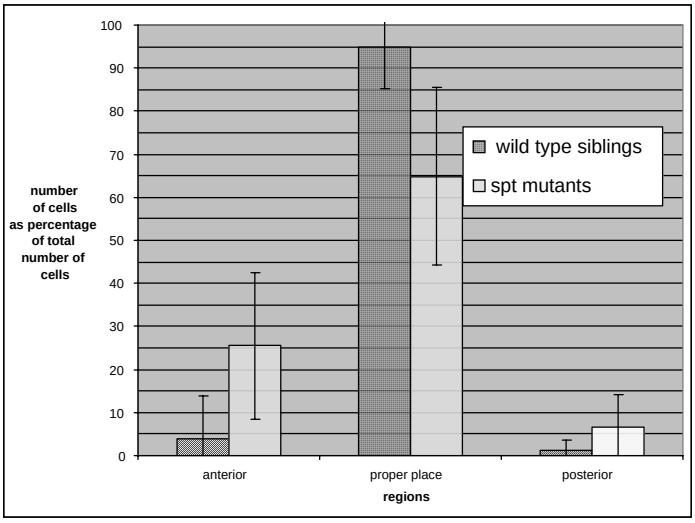
To perform the germ cell counting, three regions were defined. Cells located from the anterior to the middle of the yolk extension were considered as "OK". The other two regions are too far anterior and too far posterior. 13 *spt* mutant embryos and 11 wild type siblings were counted.



First, the counting clarified that *spt* mutant embryos do not have less or more germ cells (average 21,7 cells per embryo) than the wild type siblings (average 21,0 cells per embryo). 25% of the cells in *spt* mutant embryos, but also 4% in the wild type siblings are found too much anterior in the head whereas 7% (compared to 1% in wild type) end up in the tail. The data is summarized in figure 6.

Figure 6: Distribution of germ cells in spt mutant embryos and wild type siblings

The figure shows the distribution of germ cells in *spt* mutant embryos compared to wild type siblings. The bars show the number of cells per region as percentage of the total number of cells.



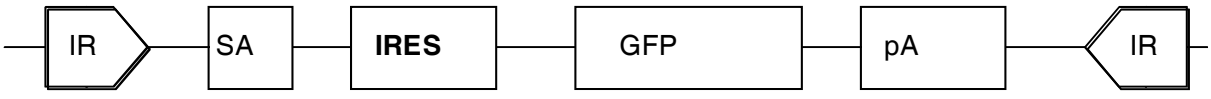
4.2 Use of IRES sequences for transposon mediated insertional mutagenesis

4.2.1 IRES as an element of gene trap cassettes

Gene trap cassettes usually have two functions: First they randomly integrate into the genome, and in case of hitting the open reading frame of a gene they disrupt the function of the encoded protein. Second in most cases the gene trap cassette contains a reporter gene which after insertion relies on the transcription from the endogenous gene for its expression (Sundaresan et al., 1995). This way the expression pattern of the "trapped" gene is marked.

A simple gene trap cassette is schematically shown in figure 7. The cassette itself does not contain the transposase but the two inverted repeats as recognition sequences. Due to that fact it is considered as nonautonomous,

Figure 7: Simple gene trap cassette



The gene trap cassette is framed by short inverted repeats (IR) on either side, which serve as recognition sites for the transposase protein, which is not shown in this drawing. In case of integration into the intron of a gene, splicing of the reporter is avoided with the help of a splice acceptor (SA) thus maintaining the expres-

sion of GFP. An internal ribosomal entry site (IRES) should serve for initiation of GFP translation even if the cassette integrates out of frame. pA = polyadenylation site.

The gene trap cassette can insert in the ORF of a gene in three possible frames but only one out of those leads to the expression of the reporter gene. In some cases, when the integration is in-frame, the reporter gene is translated as a fusion protein linked to the truncated protein of the affected chromosomal gene. Such a fusion protein will not allways be desired. To mention only one example to clarify this point, a fusion protein of a secreted protein to GFP would not be located to defined tissues. Expressing the reporter independent of the integration frame and avoiding possible non-functional fusion proteins would make the gene trap cassette three times more effective. In order to obtain this higher efficiency of marker gene expression one would have to include an IRES element that would allow cap-independent translation.

Initiation of nearly all eukaryotic mRNAs depends on a methylated cap at the free 5' end, followed by a relatively short untranslated region before the translation initiation codon (Kozak, 1989). Cap-independent initiation was first found in viruses (reviewed in (Mountford and Smith, 1995; Witherell et al., 1995)), which use IRES as a ribosome landing pad (Pelletier and Sonenberg, 1988). Some viruses inactivate the essential cap-binding complex and hence capture the cellular translational apparatus for their own use. Other viruses, e.g. encephalomyocarditis viruses do not inhibit cellular translation and their IRES sequences function efficiently in competition with cap-mediated initiation (Mountford and Smith, 1995). Importantly, the function of IRES does not depend on viral gene products (Jang and Wimmer, 1990).

In comparison with viral IRESs, relatively little is known about cellular nonviral IRESs. But examples for such elements do exist: IRES sequences were found in *Drosophila antennapedia* and *ultrabithorax* genes (Ye et al., 1997), human *Fgf2* gene (Vagner et al., 1995) and mouse vascular endothelial growth factor (Stein et al., 1998). The small number of published eukaryotic IRESs may be due to the fact that IRESs cannot be identified by sequence homology. Known IRES have been functionally defined, and so far no conserved primary sequence features have been found (Mountford and Smith, 1995). The only common features among IRESs are secondary RNA structures, important for translational initiation.

So far no zebrafish endogenous IRES which could be included into a gene trap cassette has been identified. Therefore an assay had to be established to test several IRESs for their functionality in zebrafish.

4.2.2 Choice of different IRESs

Three different IRES sequences where chosen to be tested in zebrafish. Two of them are of viral origin and the third was cloned from the mouse VEGF gene. In order to obtain viral IRESs, which display different mechanisms of ribosome attraction, the viral IRESs used in this study come from different virus families.

EMCV IRES

IRES elements were first discovered in picornavirus mRNAs which are naturally uncapped but nonetheless are efficiently translated (Jang et al., 1988). The EMCV IRES (encephalomyocarditis virus IRES) is a member of the picornaviral IRESs that have been the ones most extensively characterized up to now. Together with the foot and mouth disease virus in cell culture the EMCV IRES is the most efficient IRES element among all picornaviruses (Borman et al., 1997). In addition, it has been shown that the EMCV IRES is sufficient to initiate transcription of a bioactive gene *in vivo* in chicken (Ghattas et al., 1991).

It is interesting that different members of the picarnoviruses have different mechanisms for the utilization of the host protein machinery and selection of one of the multiple present AUG start codons (Gan et al., 1998). Most viruses shut down the cap-dependent translation by modifying the initiation factor eIF4G with viral proteases. Subsequently this factor is no longer able to mediate the cap-dependent translation, but it is still sufficient to participate in internal initiation, although the mechanism is not clear (summarized in (Gan et al., 1998)). EMCV and other members of the cardiovirus subfamily do not make use of this mechanism and therefore have to compete with mRNAs with normal 5'cap for ribosomes (Mountford and Smith, 1995). Naturally the 11^{th} ATG codon serves as initiation codon. Changes introduced by Ghattas (Ghattas et al., 1991) mutated the 11^{th} AUG to CTT so that the 12^{th} AUG serves as starting point.

BVDV IRES

Bovine viral diarrhea virus, a cattle pathogen, is a member of another family of animal viruses, the *Flaviviridae* (Chon et al., 1998) and closely related to hepatitis C virus (Poole et al., 1995). Within the *Flaviviridae* they belong to the subfamily of animal pestiviruses (Le et al., 1998). In contrast to internal translation initiation in picornaviruses, which depends on numerous interacting initiation fators, the mode of internal entry of pestiviruses and hepaciviruses resembles prokaryotic translational initiation (Chon et al., 1998). Seven ATG start codons are present in the BVDV IRES sequence and the most 3'one is used for translational initiation (Raul Andino personal communication).

With a length of 385 bp the BVDV IRES is longer than the VEGF IRES, but about 200 bp shorter than the EMCV IRES. The BVDV IRES was amplified by PCR from the BLP plasmid, obtained from Raul Andino, University of California San Francisco.

VEGF IRES

Vascular endothelial growth factor is a hypoxia inducible growth factor that is upregulated in circumstances of oxygen shortage in contrast with overall inhibition of cellular protein synthesis under these conditions (Stein et al., 1998). Together with the cumbersome structure of the 5' UTR that is incompatible with ribosome scanning, the normal way of translational initiation, this physiological requirement points to an alternative mechanism of VEGF translation. Examinations of the 1014 BP 5' UTR revealed an IRES sequence (Miller et al., 1998). Elimination of the majority (851 nucleotides) of the 5'UTR generated a significantly more potent IRES (Stein et al., 1998). This eukaryotic IRES element which is 163 BP long, was amplified by PCR from mouse day 12,5 cDNA.

4.2.3. Experimental design

The experiment was designed in a way that would make it possible to decide, which of the three chosen IRESs can function in zebrafish, and to establish a method to check other IRESs as well. The experiment should show which level of protein translation could be obtained by specific IRES mediated ribosome attraction as compared to the normal cellular mechanism of 5' cap-mediated initiation and therefore makes different IRESs comparable.

So far experiments done to examine the functionality of different IRES sequences were mostly based on dicistronic constructs. Usually the expression of the first gene is 5'cap-dependent, whereas the second should be translated with the help of the intercistronic IRES. The translation of the second gene can be monitored using different markers. Examples are *in vitro* translation with subsequent detection of chloramphenicol acetyl transferase (CAT) protein as a reporter (Witherell et al., 1995), cell culture experiments using transfection and a neomycine resistance gene under the control of the IRES in order to monitor its activity (Havenga et al., 1998), or cell culture using reporter genes like luciferase or secreted alkaline phosphatase (Lopez de Quinto and Martinez-Salas, 1998; Stein et al., 1998). *In vivo* test systems for zebrafish have not been described yet.

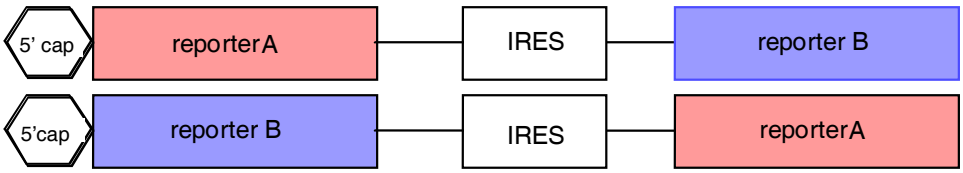
Experiments which rely on IRES dependent expression of resistance genes, like neomycine resistance (neo^r) in cell culture followed by drug selection, can clearly demonstrate whether an IRES functions or not, but makes the quantification of the activity rather difficult. Furthermore, cell cultures relies on one cell line, whereas the IRES inserted into a gene trap cassette should work in every

tissue. Since we are interested in IRES mediated gene translation in zebrafish embryos, we decided to monitor IRES activity in developing embryos.

The possibility of injecting mRNA in zebrafish embryos younger than the 4-cell stage and to detect protein production afterwards allows an easily quantifiable experiment to be performed. The experimental steps are listed below:

- Definition of two marker genes, which can be translated in zebrafish embryos without showing harmful effects on normal development.
- Developing an assay to extract the reporter proteins and to quantify their amount.
- Cloning of constructs with the three different IRES sequences between the markers. In order to have a control, two constructs should be prepared with marker A upstream and B downstream of each IRES and vice versa (see figure 8); the cloning vector should allow *in vitro* mRNA transcription as well as DNA synthesis *in vivo* by a promoter, which functions in zebrafish.
- *In vitro* transcription of the 6 different mRNAs.
- Injection of mRNA into zebrafish embryos and culturing them until detectable protein production occurs.
- Injection of DNA to prove that transcription can cross the IRES sequence in zebrafish and is still able to initiate translation of the reporter gene afterwards.

Figure 8: Schematic mRNAs to test IRES activity



Different IRES between two reporter genes are transcribed *in vitro* into mRNA and afterwards injected into zebrafish embryos in order to monitor IRES activity.

4.2.4 Reporter genes

Choice of suitable reporter genes

In vitro reporter assays refer to procedures in which the reporter protein is quantified using tissue lysates in which the reporter is expressed. These assays utilize direct quantitation of reporter protein by its enzymatic activity the level of which should reflect the level of protein synthesis.

Possible markers are CAT, luciferase, β-lactamase, and different forms of human placental alka-

line phosphatase. Most formats of the CAT assay require a relatively expensive radioactive substrate, the assays are time consuming the sensitivity of CAT assays is inferior to that of recently developed non-isotopic reporter systems (Kain, 1998). For this reasons CAT was excluded as well as luciferase, which requires expensive substrate and luminometer equipment.

The alkaline phosphatase is most commonly used in its secreted form (SEAP) in cell culture and the medium is assayed for activity(Berger et al., 1988). As PLAP is not significantly affected by heating to 65^0 C for 5 minutes so that endogenous alkaline phosphatase activity can be eliminated (Berger et al., 1987). The activity is detected by a colorimetric procedure, which is fast, inexpensive and simple to perform but is not very sensitive (Kain, 1998). In order to make the assay as sensitive as possible, SEAP, PLAP and a third truncated form of the PLAP were tested in zebrafish (see below).

The second reporter gene is β-lactamase, which is also easy to detect (O'Callaghan et al., 1972). The enzyme is able to cleave a cephalosporin thereby producing a measurable change in optical density.

The assays for alkaline phosphatase and ß-lactamase

In order to perform the measurements of two reporter proteins derived from one bicistronic construct two problems had to be solved before performing the actual experiments:

First, an extraction buffer, which efficiently elutes the reporter proteins out of the embryonic tissues, had to be designed and tested. Second, the assays had to be modified in a way, that the activity of both markers can be detected from the same lysate. In order to test the two enzymatic assays, preliminary experiments were performed mainly with the enzymes CIAP (calf intestinal alkaline phosphatase) and penicillinase (see materials). The following protocols served as a starting point:

β-lactamase assay: The activity of β-lactamase was determined as previously described (O'Callaghan et al., 1972). Briefly, 95 µl of a 0,05 M phosphate buffer at pH 7.0 containing the substrate nitrocefin were assembled with 5 µl of diluted penicillinase or embryo lysate. Nitrocefin was used in a dilution with an OD of 1,75 at 386 nm. Upon hydrolysation with β-lactamase the substrate starts to absorb with a maximum at 482 nm. This change was measured with a 490 nm filter

Alkaline phosphatase assay: The activity of alkaline phosphatase was determined as previously described (Stein et al., 1998). Briefly, 5 µl of diluted enzyme or embryo lysate were added to 95 µl alkaline phosphatase buffer (1M dietha-

nolamin (ph 9,8), 0,5 mM $MgCl_2$) in a 96 well microplate reader plate. Unlike in the described protocol 100 mM pH 9,5 tris was added to the alkaline phosphatase buffer to override the pH of the extraction buffer. The reaction was initiated by adding 20 µl of 120 mM p-nitrophenyl phosphate. The absorbance change was detected using a 405 nm filter.

Extraction buffer:

Extraction buffers are not used in the common SEAP detecting procedures, as this enzyme is released into the culture medium and does not require preparation of lysates. The same is true for ß-lactamase, which is originally a secreted protein. In contrast, the different forms of alkaline phosphatase (truncated PLAP, PLAP) were expected to be trapped in various cell compartments (see testing different variants of human placental alkaline phosphatase below). β-lactamase had to be eluted from the embryonic tissue, too.

It was therefore required to develope an extraction buffer in which the embryos were homogenized in order to get the enzymes into the solution. Homogenization was performed in a 1,5 ml eppendorf tube using 1,5 µl buffer per embryo and a plastic pistil. The buffer finally contained 50 mM NaCL, 5 mM EDTA, 0,5% Triton and 25 mM Tris. Different pH conditions were tested with the enzymes CIAP and penicillinase (see below). Likewise, different Triton concentrations were examined (data not shown).

In a first step test measurements were performed with the available enzymes CIAP and penicillinase to show that the kinetics of the colorimetric assays depends on the total amount of enzyme in the reaction. This can be measured by the change in optical density over time resulted by different enzyme concentrations.

4.2.5 Optimizing the reporter gene assays

Before doing the actual experiments both assays were modified in a way, that they can be performed using the same embryo lysate and displaying maximal activity.

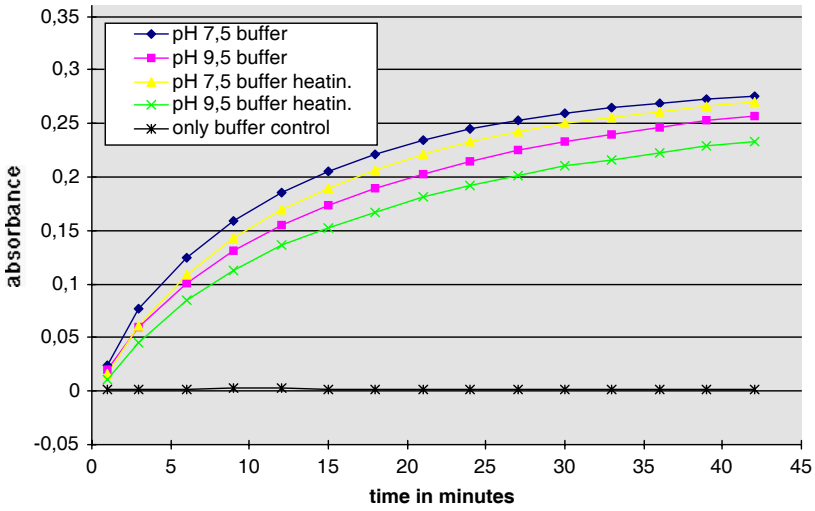
pH of extraction buffer and heat inactivation

As the β-lactamase assay and the alkaline phosphatase assay do not require the same pH the question

arose, whether the pH of the extraction buffer, and subsequently the pH of the embryo lysate, has any harmful effect to one of the two assays. In addition, the whole lysate had to be heat inactivated to eliminate endogenous alkaline phosphatases. Therefore, the heat sensitivity and the pH sensitivity of β-lactamase had to be tested. Penicillinase (see materials) was diluted 1:200 in extraction buffer at pH 9,5 and at pH 7,5. For both solutions measurements of the activity were taken with and without heat inactivation.

Figure 9: Testing penicillinase with different extraction methods

Penicillinase was diluted 1:200 in extraction buffer ph 7,5 and ph 9,5 and tested for the enzymatic activity with and without heat inactivation (heat inactivation 5 min 65 ^{0}C). The control contains only the buffer and the substrate of the colorimetric reaction without the enzyme.



The graph (figure 9) shows that penicillinase works best with an extraction buffer of pH 7,5 and no heat inactivation; however, a more basic buffer and the heat inactivation have only mild effects on the protein acitivity.

Testing different variants of human placental alkaline phosphatase

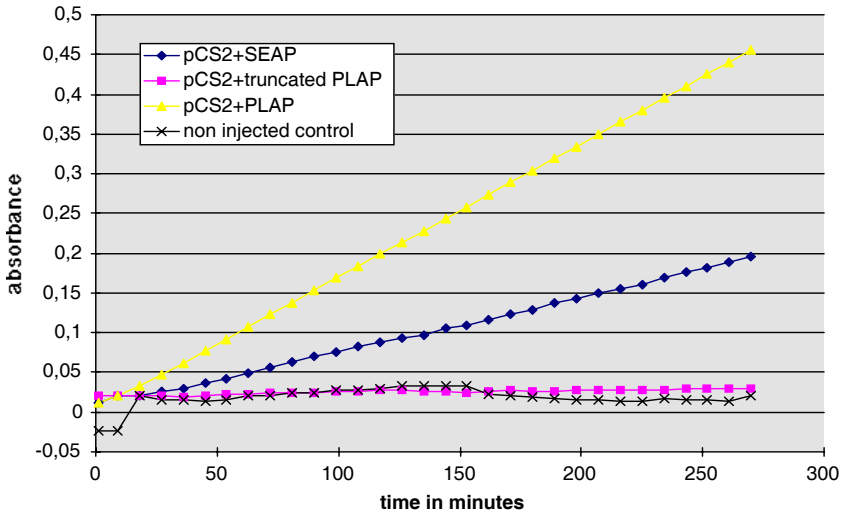
PLAP is normally anchored to the outer surface of the plasma membrane of cells by a phosphatidy-linositol-glycan (PI-G) anchor after removal of a caboxy-terminal (3') peptide (29 amino acids) from the nascent enzyme (Berger et al., 1989). The anchoring process takes place in the luminal site of the rough endoplasmatic reticulum membrane. The PI-G tailed protein then proceeds through the re-maining elements of the cellular translocation system in order to arrive at the plasma membrane (Berger et al., 1989). Truncating the PLAP by its 24 C-terminal aminoacids results in an efficiently secreted enzyme called secreted PLAP or SEAP (Berger et al., 1988). This reporter is widely used in cell culture as its activity can be detected in the culture medium. In a zebrafish assay it may not be released into the culture medium but reside in intracellular spaces of the tissue. The idea behind test-ing SEAP in zebrafish was that it might be easier to bring this enzyme into solution in order to

carry out colorimetric assays rather than membrane bound enzymes.

The 21 N-terminal amino acids of PLAP serve as a hydrophobic signal sequence (Millan, 1986), which is cleaved off to allow the protein to leave the cytoplasma and enter the endoplasmatic reticulum. Although there are no reports of proper enzymatic function in terms of truncating the signal sequence, such a truncated and non membrane bound PLAP was included in the test of different alkaline phosphatases and called truncated PLAP.

The three enzymes were amplified by PCR and cloned into the expression vector pCS2+. The truncated PLAP was amplified using 980901AP1up and 980901AP1down as primers, the SEAP with 981203PLAPfull5' and 981203SEAP3', the PLAP with 981203PLAPfull5' and 981203PLAPfull3' (primer sequences are given in section 2.5.1). Capped mRNAs were produced using the SP6 promoter.

Figure 10: Activity of different alkaline phosphatases 12 h after injection



Embryos were injected with 200 ng/µl mRNA of each construct and cultured for 12 h. 10 embryos of each injection and 10 non-injected embryos were smashed in 15 µl extraction buffer pH 9,5 each and heat inactivated. The total protein concentration was determined by Bradford assay. As all samples showed nearly the same protein concentrationc dilution to exact values was not performed. Afterwards 5 µl per sample were examined for alkaline phosphatase activity.
Except for the data for the non-injected, all values are calculated by the average of two independent measurements. To make sure that the used buffers do not display activity one sample containing the buffers only was measured as well (data not shown).

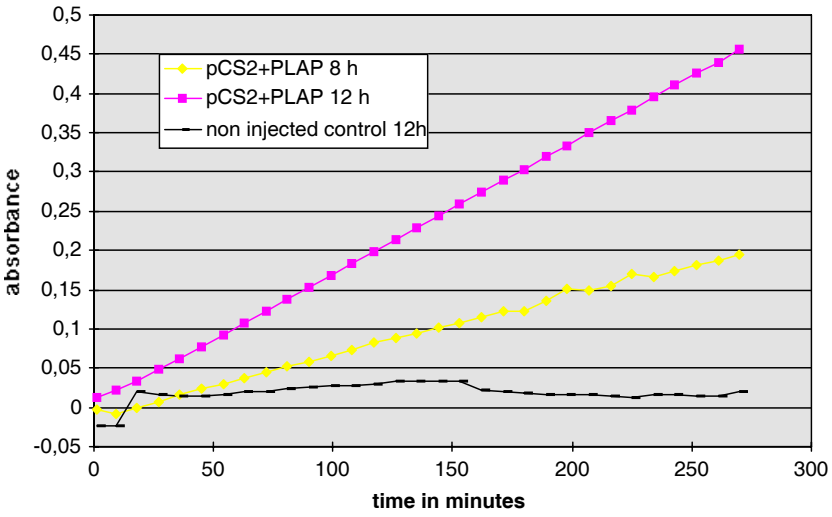
The injection of the three different forms of alkaline phosphatase reveals, that the truncated PLAP does not have any activity. This fact could be confirmed in several other injection experiments (data not shown). The SEAP reached nearly 50% of the PLAP activity (figure 10). As PLAP displayed the strongest activity in these preliminary experiments, we decided to include this form in our constructs when examining the IRES activity.

Optimal period of time between injection of the mRNA and extraction of the protein

After the injection the marker protein is being translated and is accumulating in the injected embryos while the injected mRNA is being degraded. In order to find the ideal point of time to extract the proteins two measurements at 8 h and 12 h after injection of PLAP were performed. The alkaline phosophatase assay was choosen for this test because it should be compared to the very sensitive ß-lactamase (Moore et al., 1997) the less senitive one.

Figure 11 shows that PLAP activity is much higher 12 h after injection compared to 8 h after injection. Therefore it was decided to perform the activity assays 12 hours post injection.

Figure 11: Comparison of different periods of time between injection and protein extraction



Embryos were injected with 200 ng/µl mRNA of pCS2+PLAP and cultured for 8 h or 12 h respectively. 10 embryos of each injection and 10 non-injected embryos were homogenized in 15 µl extraction buffer pH 9,5 each and heat inactivated. The total protein concentration was determined by Bradford assay. As all samples showed nearly the same protein concentration dilution to exact values was not performed. Afterwards 5 µl per sample were examined for alkaline phosphatase activity. Except for the data for the non-injected ones, all values are build by the average of two independent measurements. To ensure that the used buffers do not display activity one sample containing the buffers only was measured as well (data not shown).

4.2.6 Plasmid construction

The construction of the bicistronic constructs is outlined in figure 13. Altogether 6 different constructs were produced in the expression plasmid pCS2+, two for each IRES with β-lactamase upstream and PLAP downstream of the IRES and vice versa.

It is important to note that the sequence known as Kozak-sequence (Kozak, 1987) around the start codon plays an important role in translational initiation. As shown in figure 12 the two reporter genes have nearly the same sequences around the start ATG codon for both positions upstream and downstream of the IRES.

Figure 12: Comparison of the sequences of the reporter genes around the start ATG with the ideal sequence

REPORTERGENE	SEQUENCE
β-lactamase upstream of the IRES	TATATC**ATG**G
β-lactamase downstream of the IRES	GATATC**ATG**G
PLAP upstream of the IRES	GATATC**ATG**C
PLAP downstream of the IRES	GATATC**ATG**C
Ideal Kozak sequence	CCRCC**ATG**G

The start codon is printed in bold letters, the most important bases of the Kozak sequence in red. R stands for adenin or guanin. Those parts of the reporter gene sequences, which fit to the ideal sequence, are underlined.

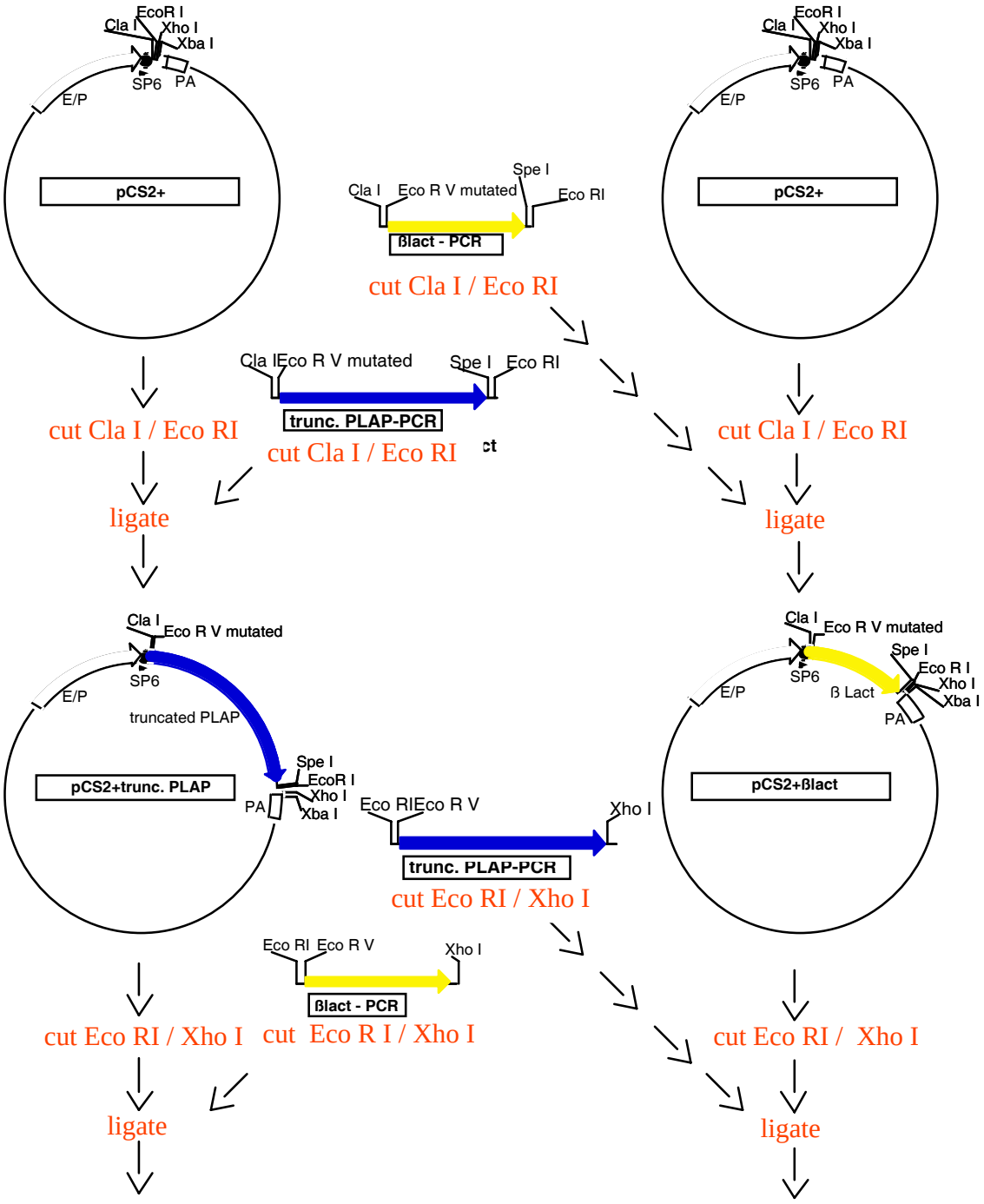
Therefore the influence of the sequence around the start codon on the attraction of ribosomes should be nearly the same for all reporter genes, thus making an unbiased comparison of the reporter activity possible. In addition, it is important to notice that the two 3' primers used to amplify the reporter genes, which were included upstream of the IRES into the construct, were carefully designed. They contained stop codons in each of the three possible reading frames, to make sure that the ribosomes fall off the mRNA instead of scanning along and being reassembled at the IRES sequence (primers see materials section 2.5.1).

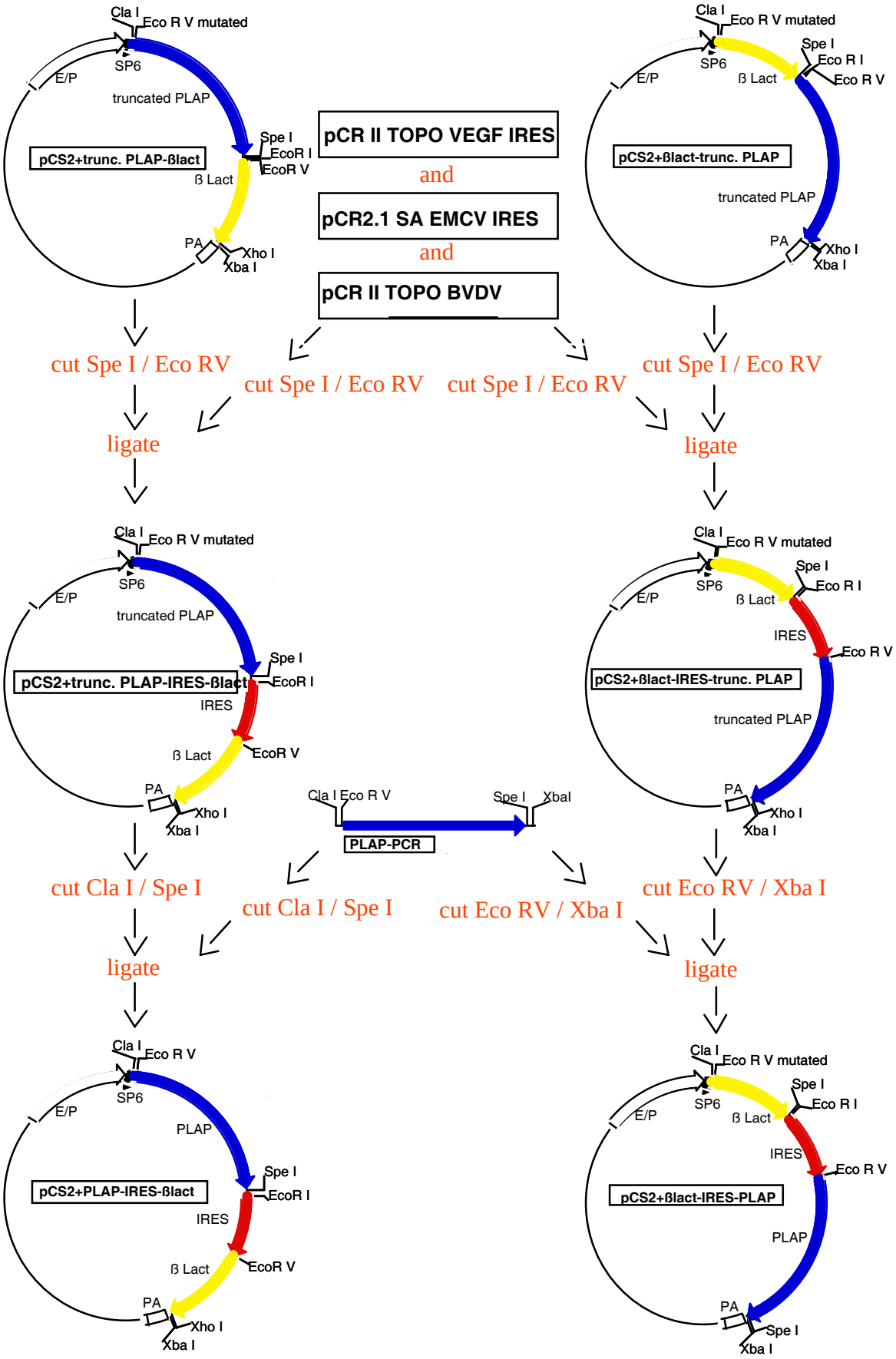
The cloning products were checked by digests and by sequencing. Sequencing was performed starting from the SP6, T3, and T7 promoter as well as from internal sequences with specially designed sequencing primers (see section 2.5.1).

Figure 13: Schematic construction of the 6 plasmids used to test the 3 IRESs

The plasimds, which served as PCR-template for the reporter genes and the IRESs, are not shown. pCS2+ contains a T7 promoter upstream and a T3 promoter downstream of the SV 40 polyadenylation site (PA), which are not shown. Additionally pCS2+ contains the gene for ampicilline resistance and a F1 origin (not shown).

E/P = CMV enhancer promoter; PA = SV 40 polyadenylation site; β lact = β-lactamase




pCR II TOPO VEGF IRES
and
pCR2.1 SA EMCV IRES
and
pCR II TOPO BVDV
pCS2+trunc. PLAP-ßlact
pCS2+ßlact-trunc. PLAP
pCS2+trunc. PLAP-IRES-ßlact
pCS2+ßlact-IRES-trunc. PLAP
pCS2+PLAP-IRES-ßlact
pCS2+ßlact-IRES-PLAP
PLAP-PCR
cut Spe I / Eco RV
cut Cla I / Spe I
cut Eco RV / Xba I
ligate
Cla I
Eco R V mutated
SP6
E/P
truncated PLAP
Spe I
EcoR I
EcoR V
ß Lact
PA
Xho I
Xba I
IRES
PLAP
Cla I Eco R V
Spe I
XbaI

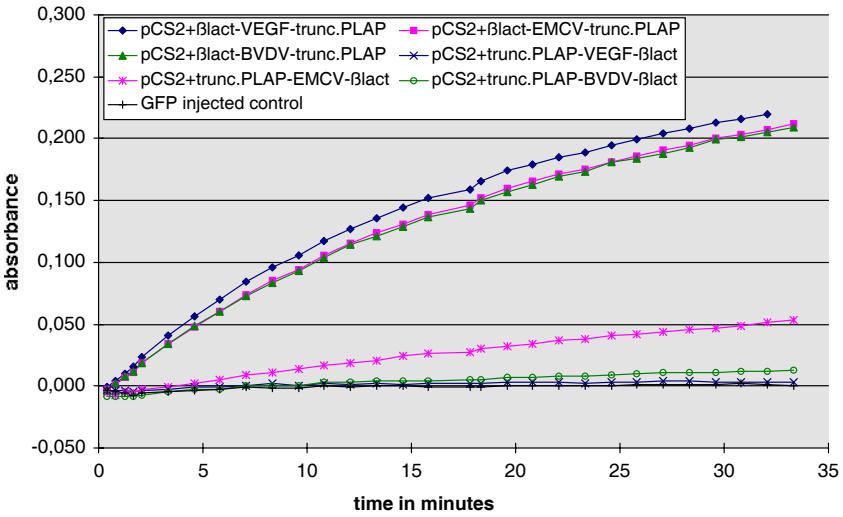
4.2.7 mRNA injection

Detecting ß-lactamase

As a starting point and due to the fact, that the first becistronic constructs contained the truncated alkaline phosphatase, a first assay was done detecting the activity of ß-lactamase only. 6 mRNAs, transcribed from constructs with the three different IRESs either with ß-lactamase downstream or upstream (cloning of the constructs see section 4.2.6), were injected into embryos up to 4-cell stage (100 ng/µl). The measurement was performed at 50% epiboly using embryos from which the yolk had been removed.

Figure 14: Detection of ß-lactamase after mRNA injection (50% epiboly)

15 embryos were homogenized in 25 µl extraction buffer with pH 7. The total protein amount was determined by Bradford assay.

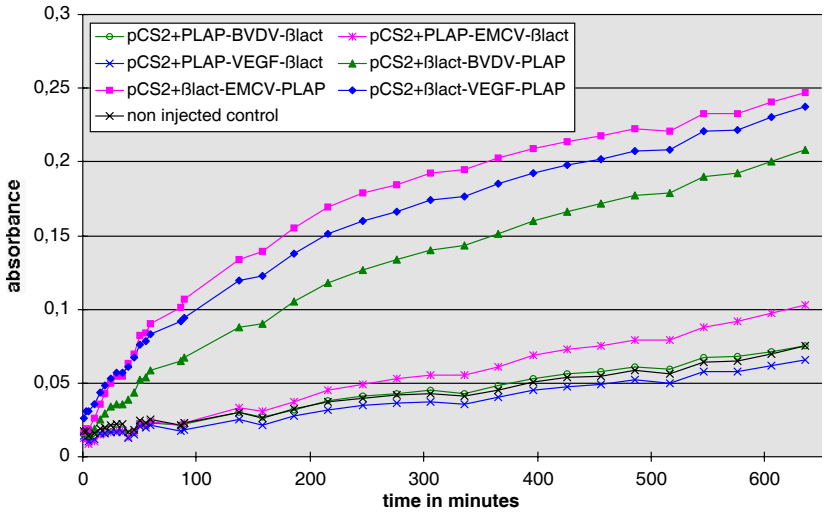


This assay demonstrated, that ß-lactamase activity can be detected in lysates of injected embryos. Furthermore it pointed to the fact, that the EMCV IRES can work in zebrafish, but not the BVDV or the VEGF IRES. The IRES depended translation reaches a level of 18% compared to that of the 5'cap (see figure14). To demonstrate that both reporter genes are translated from one mRNA the reporterconstructs had to be modified.

Detecting both reporters in one assay

To be able to decide which of the IRES can work in zebrafish, 6 mRNAs transcribed from constructs with the three different IRESs were injected into embryos younger then 4-cell stage (200 ng/µl). 12 hours after the injection the essays for ß-lactamase and alkaline phosphatase were performed.

Figure 15: Detection of ß-lactamase 12 h after mRNA injection



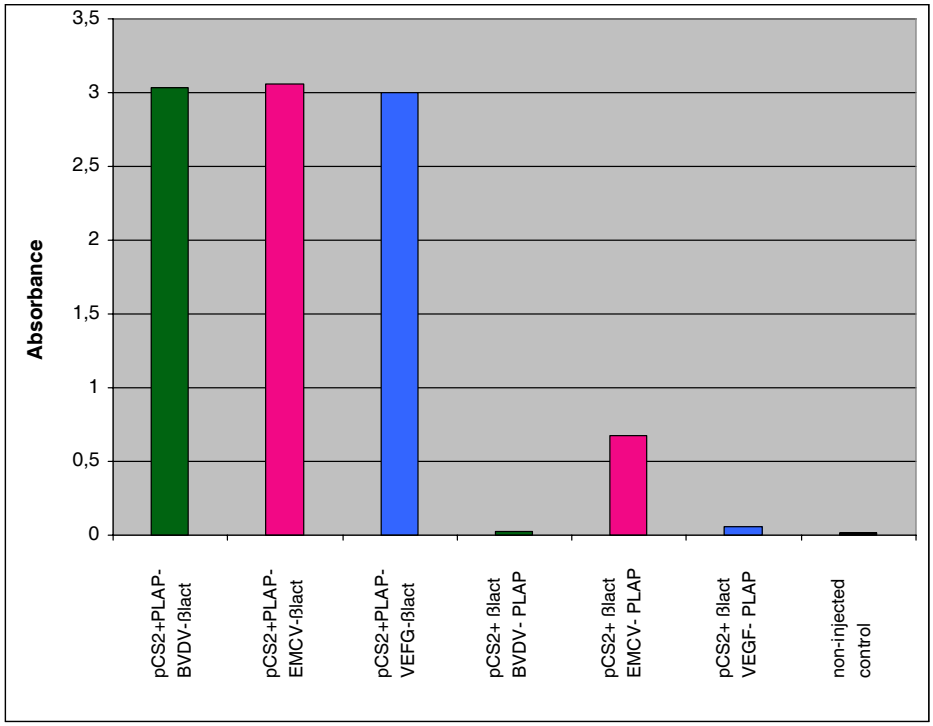
Embryos were injected with 200 ng/µl mRNA of each construct and cultured for 12 h. 30 embryos of each injection (only 15 embryos pCS2+PLAP-BVDV-ßlact injected) and 30 non-injected embryos were smashed in 1,5 µl extraction buffer pH 9,5/embryo each and heat inactivated. The total protein concentration was determined by Bradford assay. As all samples showed nearly the same protein concentration dilution to exact values was not performed. Afterwards 5 µl per sample were examined for ß-lactamase activity.

Except for the data for the pCS2+PLAP-BVDV-ßlact injected ones all values are calculated by the average of two independent measurements. To make sure that the used buffers do not display activity, one sample containing the buffers only was measured as well (data not shown).

The same lysat that was produced to measure the activity of ß-lactamase was used to perform the assay for alkaline phosphatase. The reaction was started but continous measuring of alkaline phosphatase was not possible, as the microplate reader was used for measuring the ß-lactamase assay at the same time. Therefore a measurement of the alkaline phosphatase assay was taken directly after the start of the reaction to prove that all wells displayed no activity. The plate was kept in darkness and a second measurement was taken 625 minutes after the start of the reaction. The results are shown in figure 16.

Figure 16: Detection of alkaline phosphatase 12 h after mRNA injection

The measurement to demonstrate the activity of PLAP was taken 625 minutes after the start of the reaction.



In both assays the gene translated from the 5'cap displays strong activity, but the level of activity between the constructs with the different IRES varies more in the ß-lactamase reaction. With both markers the BVDV and the VEGF IRES do not show significantly more activity than the non-injected control. The EMCV IRES is able to initiate translation of the downstream marker irrespectively of the kind of marker used. ß-lactamase is produced starting from the IRES with 16% of the level of the protein translation initiated by the 5' cap (figure 15) and PLAP with 21,62% (figure 16) respectively.

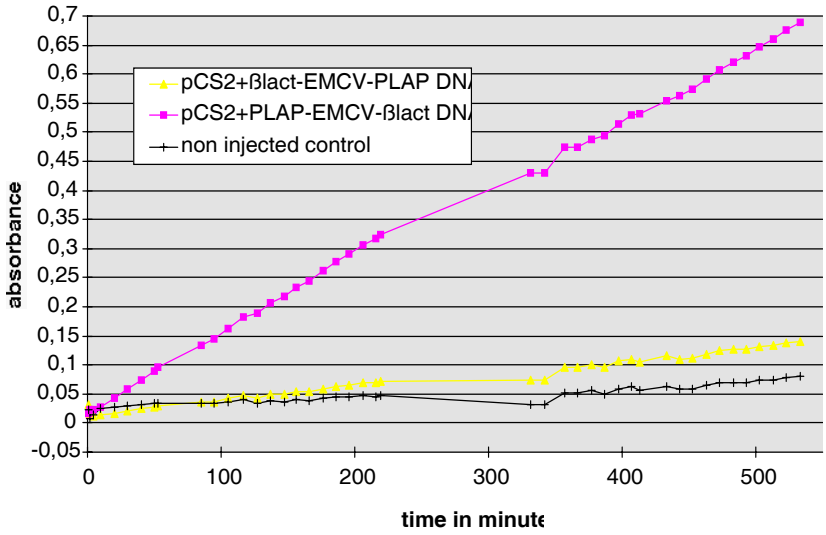
This data confirms the data from the first test with the ß-lactamase assay, which demonstrated that the EMCV IRES works with 18%.

4.2.8 DNA injection

If the IRES is used in a gene trap cassette, it will integrate as DNA into the genome of zebrafish. For this reason it is not sufficient to show that in a bicistronic mRNA construct the expression of the second gene can be driven by IRES sequences. The IRES has to be crossed by the trancriptional machinery *in vivo* in order to get expression of the marker gene.

Since in the mRNA injections only the constructs with EMCV IRES showed activity, only these constructs were tested at the DNA level. Production of protein after DNA injection requires more time than is the case for the mRNA injection, because the message has to be transcribed to mRNA first. For that reason, DNA injected embryos were not examined after 12 h but cultured overnight and examined 24 h after injection. The activity for alkaline phosphatase 24 h after injection is shown in Figure 17.

Figure17: Detection of PLAP 24 h after DNA injection



Embryos were injected with 20 ng/µl DNA at one cell stage. 24 h after injection 30 embryos of each injection and 20 non injected embryos were smashed in extraction buffer (1,5µl buffer/embryo).

The total protein amount was checked by Bradford assay after heat inactivation. The pCS2+ßlact-EMCV-PLAP construct had a slightly lower concentration (92%) than the construct with PLAP upstream of the IRES. Between 220 and 320 minutes no measurements were taken, because the Microplate Reader was used for measuring ß-lactmase activity.

The activity of PLAP downstream of EMCV IRES reached over 10% of the enzyme transcribed and translated from the 5' cap. Considering that the protein concentration in this sample is even lower than in pCS2+PLAP-EMCV-ßlact, this result proves that the EMCV IRES DNA can be translated and transcribed in the zebrafish system, albeit to a low level.

5. DISCUSSION

5.1 Germ cell migration in mesoderm mutants

5.1.1 Proper germ cell migration requires intact paraxial mesoderm, but is not affected by loss of axial mesoderm tissue

Germ cells migrate abnormally in embryos mutant for the *spt* gene, but show a normal migration pattern in *flh* or *ntl* mutants. In *spt* mutant embryos a portion of the primordial germ cells are found in ectopic locations along the anterior-posterior axis. In *spt* mutants 25% of the PGCs arrive at a distinct region in the head between the midbrain-hindbrain boundary and the otic vesicle whereas 7% end up at various positions in the tail. These findings are consistent with the view that somatic tissues are involved in directing the PGCs towards their final destination in zebrafish. More specifically, proper differentiation of the paraxial mesoderm, which is affected in *spt* mutants, is important for proper germ cell migration. In contrast to that, defects in the axial mesoderm, which develops partially in *ntl* mutants, or is lacking completely in *flh* mutants, does not affect the proper migration of PGCs towards their final location on either side of the midline.

The total number of germ cells in *spt* mutant embryos (those located in the normal location and the ectopic cells) is similar to the total number of cells in wild type siblings. This finding supports the notion that the ectopic germ cells in *spt* initially belonged to the four original cell clusters, and have migrated abnormally during gastrulation.

5.1.2 Ectopic germ cells in *spt*

Different models can be proposed to explain the ectopic location of the germ cells in *spt* mutant embryos.

The four clusters of PGCs, which are formed during the cleavage stages can be detected at shield stage spaced around the embryo and usually near the blastoderm margin. The orientation of these clusters relative to the embryonic shield, or the dorsal aspect of the embryo is random (Yoon et al., 1997). During early somitogenesis two germ cell clusters are observed at the level of the third to fifth somite. The precise positioning of the germ cell clusters at early somitogenesis suggests the exis-

tence of an active guidance mechanism directing germ cells to the position of the cluster, irrespective of their original position which differs from embryo to embryo.

One can think of two different explanations why at least some germ cells seem to be unable to arrive at the cluster region. As *spt* embryos exhibit paraxial mesodem defects, the activity of a putative attracting signal originating from the future gonadel region might be reduced. An example for the existence of such a mechanism is the *Drosophila* HMG-CoA reductase which is expressed in the gonadal mesoderm and functions in attracting germ cells in *Drosophila* (Van Doren et al., 1998). Reduction in the activity of such an attracting signal could lead to mislocalization of cells that are originally located away from this signal. Another explanation for the *spt* germ cell phenotype is that the primordial germ cells may need a correctly organized environment for their migration. Preceding gastrulation, the future mesodermal cells in the dorsal marginal zone acquire a cohesive cell behavior, which depends on the function of the *spt* gene (Warga and Nüsslein-Volhard, 1998). This cell compaction may restrict early mixing and dispersal of dorsal cells. Thus in *spt* mutant embryos germ cells of clusters which lay in the shield region may be more likely to disperse and may face problems when they migrate to more posterior levels, due to loss of cohesive cell behavior in this region.

It is shown, that *spt* acts cell-autonomously and that wild type cells transplanted to *spt* mutant embryos follow their normal fate and form muscle cells instead of being dragged along with the mesenchymal cells to the “spade” (Ho and Kane, 1990). Therefore the possibility that germ cells which end up in the tail are dragged along with the stream of “spade” forming cells is less likely. This explanation would also require that all ectopic cells end in the “spade” region, and not all over the tail as they do. With the movement of mesodermal precursor to the tail a whole population of cells which might provide an attractive signal for the germe cells is missing in this region. This might be the reason, why a part of the PGCs in this region lose their migration pathway and end up in various region.

5.1.3 Further experiments

To address the question why the ectopic cells in *spt* end up in the head region and in the tail a more detailed analysis of stages later than shield and earlier than 24 hpf would be necessary. Using markers for distinct regions in the hindbrain could help to precisely define the region were the ectopic cells end up.

The fact that *spt* embryos in total do not have more germ cells than the wild type siblings, suggests that the loss of cells in the main cluster does not lead to extra cell divisions during the first 24 hour of development. It would be interesting to follow the ectopic cells and check, wether they will die or transfate to the new tissue, which surrounds them. For this purpose it would be necessary to

follow the fate of the ectopic cells using molecular markers specific for the germ cell and to determine if the ectopic cells express markers normally expressed by cells located in the region of the ectopic clusters.

5.2. Use of IRES sequences for transposon mediated insertional mutagenesis

5.2.1 EMCV IRES can work in zebrafish

Using becistronic reporter constructs it could be shown that the EMCV IRES can function in the zebrafish system, while the BVDV IRES and the VEGF IRES showed no activity.

In mRNA injection experiments, the level of protein production of the gene cloned downstream of the IRES is at least 15% of the protein level that can be obtained when the gene is cloned at the 5' position. In DNA injection experiments, the efficiency of protein translation of the marker cloned downstream of the IRES is about 10% compared to the level obtained when the gene is placed in the 5' position.

A possible explanation why the EMCV but not the BVDV IRES can function in zebrafish is the different mechanism of translational initiation promoted by these two elements. Virus genes translated in a cap-independent manner using IRES sequence, often shut down the cap-dependent cellular translation by proteolytic cleavage of the initiation factor eIF4G. This factor functions as linker protein which joins the various factors involved in mRNA recruitment to the 40 S ribosomal subunit (Gan et al., 1998). Encephalomyocarditis viruses do not inhibit cellular translation by such a proteolytic cleavage, and their IRES sequences function efficiently in competition with the cap-mediated initiation (Mountford and Smith, 1995). Upon injection of *in vitro* transcribed mRNA as done in the experiments presented above, cap-dependent translation is not inhibited. While the EMCV IRES is still able to compete for ribosomes under these conditions, the BVDV IRES might be unable to attract ribosomes.

The mouse VEGF IRES helps to maintain the expression of a growth factor, which is important for blood vessel formation under the conditions of hypoxia when translation of other mRNAs is inhibited. 163bp of the 1014 bp 5' UTR suffices for the function as an IRES. This sequence works synergistically with the 5' cap and helps to attract ribosomes to an otherwise poorly translated mRNA (Stein et al., 1998). Therefore, the VEGF IRES is not only able to attract ribosomes under conditions of hypoxia when the overall protein synthesis is lowered and ribosomes are free, but is

also sufficient do direct ribosomes to the downstream ATG start codon under conditions of normoxia. As the VEGF IRES can function under normal oxygen levels we would expect it to function under the normoxia conditions of the experiments described in the result section. A possible explanation for the lack of activity in the case of this IRES is that several cofactors which are not present in zebrafish embryos are needed to allow translation initiation from the VEGF IRES.

5.2.2 Using an EMCV IRES in a gene trap cassette

To decide whether the EMCV IRES should be included into a gene trap cassette for an insertional mutagenesis screen, several points have to be taken into consideration. The fact that when using an IRES the translation of the reporter becomes independent of the integration frame and therefore enhances the screen threefold argues for the utilization of an IRES. In contrast, the efficiency of the IRES mediated reporter protein production will only be between 10% and 20% of the level of the translation of the gene in which the cassette integrates. This could mean that trapping genes with very low transcriptional activity will be more difficult.

In addition, it seems that the size of the cassette be transposed efficiently is limited (e.g. *sleeping beauty,* Ivics, Z. pers. com.). The size of the EMCV IRES is over 600 bp. Depending on the size of the other elements of the gene trap cassette, this size could be critical and may lead to a decreasing rate of transposition.

The fact that when IRES are used no fusion protein is produced is not always desirable. Sequences contributed by the host can direct subcellular localization of the fusion product (e.g. with GFP) and thereby provide valuable information regarding the nature of the trapped product. This information will not be obtained when IRES element is used.

5.2.3 Further experiments

In order to prove that the EMCV IRES alone is sufficient to attract ribosomes and that the reporter protein downstream of the IRES is not fused to the reporter translated from the 5’ cap, two experiments can be performed. One possibility is to inject *in vitro* synthesized uncapped RNAs of the 6 constructs with PLAP and ß-lactamase and to use the biochemical assays in order to show that with the EMCV IRES the second reporter is translated whereas almost no protein is produced from the first reporter gene. A second way to prove the activity of the EMCV IRES would be to inject mRNA and show on a protein gel of the embryo lysate that two non-fused reporter proteins are produced.

In order to find IRES sequences which work with a higher efficiency in zebrafish several approaches can be taken using the vectors and procedures described in this work. For example examin-

ing the zebrafish VEGF 5' UTR may be a possibility to find the first endogenous IRES sequence in zebrafish. This approach is facilitated by the fact that the zebrafish VEGF IRES is cloned (Liang et al., 1998). As this gene might have similar functions to the mouse VEGF gene, responding to oxygen shortage by upregulation of transcription and translation, a common translational regulation utilizing IRES sequences is possible.

In addition, one could work on optimizing the EMCV IRES by altering its sequence slightly or by modifying the distance between the IRES and the downstream reporter in order to define more efficient versions.

5.2.4 Cloning an endogenous zebrafish IRES

As mentioned above, the zebrafish VEGF gene is cloned and it contains a 586 bp long 5' UTR (Liang et al., 1998). The length of the zebrafish 5' UTR is only half the length of that of the mouse, but since the mouse VEGF IRES is only 163 bp long the zebrafish VEGF might also contain a functional IRES. An alignment of the mouse VEGF IRES to the zebrafish VEGF 5' UTR does not show any evident sequence similarity, which rules out out the possibility to clone an IRES element directly by sequence alignment.

The experimental design to evaluate the 5' UTR of the zebrafish VEGF IRES would then be to clone the whole UTR into the becistronic reporter vector first and in case activity is obtained to carry out deletion analysis in order to obtain a small element.

4.3.1 Possibility to construct becistronic messages

Unlike in prokaryotes, in eukaryotes mRNAs coding for more than one protein do not occur naturally Having an artificial tool, which allows translation of two proteins from one mRNA would make a wide range of experiments possible. For example, two genes of biological interest could be hooked up. Upon injection of *in vitro* transcribed mRNA the genes would act in exactly the same cells unlike the situation upon injection of a mixture of two mRNA, which can diffuse and be translated in a slightly different subset of cells. A second possibility would be to have a gene of biological interest and GFP on the same construct. Upon injection, the GFP would be expressed in the same cells as the other gene and yield its expression pattern allowing one to follow the fate of cells expressing the gene of interest.

The EMCV IRES could be such a tool to construct becistronic messages that work in zebrafish. Therefore, it has to be proved that the level of translational initiation allowed by the EMCV IRES is

sufficient to produce an amount of GFP that can be visualized, and that the translation pattern of the gene and GFP completely overlap.

SUMMARY

For the purpose of investigating the formation and the migration of germ cells in zebrafish two approaches were taken. First, the germ cell migration was monitored in several mutants affected in mesoderm development using the *vasa* gene as a molecular marker. It is shown, that paraxial mesoderm, which is affected in *spadetail* mutants is important for the guidance of germ cells. In contrast, germ cell migration is normal in mutants with defects in axial mesoderm (*floating head* and *no tail*) ruling out a possible role of the notochord in attracting germ cells to their proper location in zebrafish.

Second, for the purpose of building a gene trap cassette, the activity of several internal ribosomal entry sites (IRESs) was tested in the zebrafish system. An inherited problem in gene trap schemes is that only in one out of three cases the marker gene will be fused in-frame to the coding region of a trapped gene. The use of IRES sequences upstream of reporter genes can help to circumvent this problem. The internal translation start allows the production of a full-length protein irrespective of the reading frame of the trapped gene, and therefore strongly enhances the efficiency of such vectors. In addition, IRES sequences can be used to co-express two different proteins encoded on one RNA molecule. Unfortunately, so far no endogenous zebrafish IRES has been identified and IRES primary sequences are not conserved to allow screening for fish IRES.

In order to include an efficient internal ribosomal entry site in a gene trap cassette which will be used in a transposon mediated insertional mutagenesis screen, we tested the activity of IRES sequences from BVDV, EMCV and mouse VEGF 5´UTR. Expression plasmids were constructed containing a reporter gene upstream and a second reporter gene downstream of the different IRES sequences and vice versa. The translation of the first gene depends on the 5´cap-binding mechanism and can be compared to the translation of the second gene whose translation depends on the IRES activity. Injections of synthetic mRNAs and DNA coding for these dicistronic reporter constructs into zebrafish embryos allowed to measure the activity of the internal translation by colorimetric assays. Using this assay it is shown that the EMCV IRES can function in the zebrafish system at a level of 15-20% compared to the 5’ initiation.

ZUSAMMENFASSUNG

Zur Untersuchung der Bildung und Wanderung von Keimzellen im Zebrafisch wurden zwei Ansätze gewählt. Einerseits wurde die Keimzellwanderung in verschiedenen Mutanten mit Defekten in der Mesodermbildung mit Hilfe des *vasa* Genes als molekularem Marker sichtbar gemacht. Dabei konnte gezeigt werden, daß paraxiales Mesoderm, das in *spadetail* Mutanten betroffen ist, für die Lenkung und Orientierung der Keimzellwanderung eine wichtige Rolle spielt. Im Gegensatz dazu verläuft die Keimzellwanderung in Mutanten mit Defekten im axialen Mesoderm (*floating head* und *no tail*) normal. Damit scheidet im Zebrafisch eine mögliche Rolle des Notochords für die Anziehung der Keimzellen zu ihrer richtigen Position aus.

Andererseits wurde als eine Grundlage für die Klonierung einer “Gene trap”-Kassette die Aktivität verschiedener interner ribosomaler Eintrittsstellen (IRESs) im Zebrafisch getestet. Ein immer wiederkehrendes Problem bei “Gene trap”-Projekten ist nämlich, daß das Markergen nur in einem von drei Fällen mit dem Gen, in das die Insertion erfolgt ist, im Leseraster der codierenden Region fusioniert. Das Einklonieren von IRES Sequenzen stromaufwärts vom Reportergen kann helfen, dieses Problem zu umgehen. Der interne Start der Translation ermöglicht die Produktion eines vollständigen Proteins, unabhängig vom Leseraster des Genes in das die Insertion erfolgt ist, und erhöht deshalb die Effektivität solcher Vektoren. Zusätzlich können IRES Sequenzen dazu benutzt werden, zwei Proteine, die von einer RNA codiert werden, zu coexprimieren. Leider ist bislang keine endogene Zebrafisch IRES Sequenz bekannt, und IRES DNA Sequenzen weisen keine Strukturkonservierung auf, die eine systematische Suche nach Fisch IRES Sequenzen zulassen würde.

Um eine effektive IRES in eine “Gene trap”-Kassette, die für eine Transposon vermittelte systematische Isolierung von Insertionsmutanten verwendet werden soll, einschließen zu können, wurde die Aktivität der IRES Sequenzen von BVDV, EMCV und 5’UTR des Maus VEGF Genes untersucht. Dazu wurden Expressionsplasmide, die ein Reportergen stromaufwärts und ein zweites stromabwärts der unterschiedlichen IRES Sequenzen enthalten, sowie solche mit der umgekehrten Anordnung der Reportergene konstruiert. Die Translation des ersten Reporters beruht auf dem 5’-Kappen-Bindungsmechanismus und kann mit der Translation des zweiten Genes, dessen Translationsrate von der IRES-Aktivität abhängt, verglichen werden. Injektionen synthetisch hergestellter mRNA und DNA, die für diese bicistronischen Reporterkonstrukte codieren, in Zebrafischembryonen machten es möglich, die Rate der intern gestarteten Translation mit Hilfe colorimetrischer
Assays zu messen. Mit dieser Methode konnte gezeigt werden, daß die EMCV IRES im Zebrafisch mit 15-20% der Aktivität, verglichen zur Translation von der 5’ Kappe arbeiten kann.

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