ika1 and *rag1* as Markers for the Development of the Zebrafish Immune System

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Non-Scientific Abstract

The immune system is responsible for defending the body against bacteria and viruses which can cause illness. Immune cells in the blood can destroy infections, protecting animals against diseases. By studying the way the immune system works, scientists can design more effective ways to fight illnesses. One way to study the immune cells is to research how they develop in an embryo. Genes in a developing embryo are turned on and off to make some cells decide to become immune cells. Our lab is studying some of these genes in zebrafish. Because zebrafish are small and easy to study, we are using them to research genes that would be harder to study in mice or humans. In this paper, we describe cloning *ika1*, one of these genes.

Scientific Abstract

Zebrafish are useful vertebrates in which to carry out large-scale mutagenic screens to identify developmentally important genes. In order to follow the development of the zebrafish immune system, we previously isolated and characterized the recombination activating genes, *rag1* and *rag2*. This paper describes the cloning and characterization of another such marker, *ika1*, which encodes a zinc finger transcription factor necessary for early lymphocyte differentiation. Whole mount *in situ* hybridization revealed expression of *ika1* in 24 hr embryos in the intermediate cell mass, the first site of hematopoiesis (Weinstein *et al.*, 1996), and the head. We also report the continuing efforts to create transgenes with the green fluorescent protein-coding region under the control of the promoters of *rag1* and *ika1*. Fish containing these transgenes could then be mutagenized and screened quickly and easily for mutations in the immune system.

Introduction

Zebrafish are appropriate for research in developmental biology for a number of reasons. Their large brood size, relatively short generation time, and modest space requirements make them easier to breed than many other vertebrates. Also, their embryos are clear and develop outside of the mother, facilitating easy screening for visual abnormalities that might occur during development. Therefore, zebrafish are good organisms for genetics, including screens for mutations in genes affecting development. Genetic tricks such as the creation of haploids or gynogenotes could also facilitate screening (reviewed in Driever *et al.*, 1994). A large scale mutagenic screen in Boston and Tübingen, Germany, recently identified more than 400 genes important to zebrafish embryogenesis (Driever *et al.*, 1996; Haffter *et al.*, 1996).

The development of the zebrafish immune system is of particular interest to our laboratory. In order to use the full potential of zebrafish in detecting immune system mutations, it is necessary to develop markers and describe the early development of the immune system. For these purposes, the lab previously cloned the recombination activating genes, *rag1* and *rag2* (Willett *et al.*, 1997 a). These genes are expressed primarily in the thymus, a structure which becomes visible by whole mount *in situ* hybridization on day four of development (Willett *et al.*, 1997 b).

We would like to have an additional marker for lymphoid cells which is expressed earlier than the *rags*. We decided to clone *ika1*. In mice, Ikaros is a zinc finger transcription factor essential for the development of all lymphoid lineages. The gene is expressed in mice in T and B lymphocytes and natural killer cells as well as their progenitor cells (Georgopoulos *et al.*, 1992; Georgopoulos *et al.*, 1994). Thus, *ika1* is expected to be expressed somewhat earlier in the lymphoid lineages than the *rag* genes. We therefore cloned the *ika1* genomic DNA and used a PCR fragment to do *in situ* hybridization in zebrafish embryos.

This paper also describes initial attempts to isolate the promoter regions of the *rag1* and *ika1* genes and fuse them to the coding region of GM2, a variant of the green fluores-

cent protein (GFP) gene. These reporter constructs will then be used to create two lines of transgenic fish. These fish could be quickly and easily screened for expected expression of the transgenes simply by looking at them under a FITC light filter, a method which will hopefully be not only quicker, but also more sensitive than screening by *in situ* hybridization, with the additional benefit that live fish can be screened. Similar constructs with *gta1* and *gta2* have been shown to produce specific expression of the transgene, and fish containing the transgene are able to pass it through the germ line (Long *et al.*, 1997; Meng *et al.*, 1997).

Materials and Methods

Animals
Wild type *Danio rerio* embryos were obtained from Nancy Hopkins and reared in beakers in a 28°C water bath.

Plasmids and Probes
RGM2 (kindly provided by S. Lin) contains 4.7 kilobases (kb) of the *rag1* 5' region fused to the GM2 coding sequence followed by an SV40 polyadenylation signal. The backbone is pBluescript S/K- (Stratagene, La Jolla, CA). The *ika1* probe is a 600-bp PCR product generated by S. Lin using degenerate oligonucleotides on zebrafish genomic DNA. The fragment, located approximately 60 bases into exon seven, was cloned into Litmus29 (NEB, Beverly, MA), and designated IkPCR. A DNA probe was generated by random-primed labeling of the PCR fragment with [α-^{32}P] dCTP using a labeling kit (Boehringer-Mannheim, Indianapolis, IN). RNA antisense probe was transcribed from a *Bam*HI digestion of

IkPCR using T7 polymerase and labeled with digoxigenin using a labeling kit (Boehringer-Mannheim).

A λ clone containing the *rag1* and *rag2* coding regions was previously isolated (Willett *et al.*, 1997). The most 5' 3.5 kb *Sac*I fragment (relative to *rag1* transcription) of this clone was designated clone 10. To screen for *rag1* upstream sequences, we used DNA probe generated from clone 10 by random priming as described above.

Cloning *ika1* and the *rag1* 5' Region
A zebrafish genomic library was obtained from S. Statchel (University of California, Berkeley). The library is a *Sau*3A partial digest of genomic DNA from 72 hr whole fish cloned into λ Fix II (Stratagene). 500,000 plaques were lifted and probed with the insert from IkPCR. Several positive clones were obtained, one of which, IK4A, was selected for further study. The 17.6 kb insert was digested with *Sac*I and the fragments subcloned into pBluescript S/K - (Stratagene). A genomic clone containing *rag1* upstream sequences was obtained by screening a λ Fix II genomic library (Stratagene) with the insert from clone 10. 500,000 plaques were lifted, and several positive clones were obtained, one of which was selected for further study.

Southern Blotting
One μg of the *ika1* λ clone was cut with various restriction enzymes, run on a 1% agarose gel, and transferred to a Nytran nylon membrane (Schleisher and Schull, Keene, NH) in 3 M NaCl and 8 mM NaOH. The membrane was probed with the IkPCR DNA probe and hybridized overnight at 65°C in 5X SSC [1X SSC: 0.15 M NaCl, 15 mM sodium citrate, pH 7.0], 1% blocking reagent (Boehringer-Mannheim), .1% N-

Fig. 1. Nucleotide and translated amino acid sequence of the IkPCR insert. The insert was sequenced from primers in the vector.

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IKpcr    accagagctccctaggttctagaaccggtgacgtctcccatggtgaagcttggatcccat 60
                                                          S   H   2
IKpcr    gtgatggatcaggccatcaacagtgcaattagctatctgggtgcagagtccttgcggcct 120
          V   M   D   Q   A   I   N   S   A   I   S   Y   L   G   A   E   S   L   R   P  22
IKpcr    ctggttcagacctctcctgggtccgccgacatggtggtcagccctctatacaacctgcac 180
          L   V   Q   T   S   P   G   S   A   D   M   V   V   S   P   L   Y   N   L   H  42
IKpcr    aagtcacaaacagctgaaggcaatggtgtttctgctaaagacagcgccgcagagcacctt 240
          K   S   Q   T   A   E   G   N   G   V   S   A   K   D   S   A   A   E   H   L  62
IKpcr    ctcctactctctaagtccaaatccgcctctgttgacaaagacggttcccccagtcccagc 300
          L   L   L   S   K   S   K   S   A   S   V   D   K   D   G   S   P   S   P   S  82
IKpcr    cggcaggattccactgacactgagagcaacaacgaggagcgttcggccggggtaagcgga 360
          R   Q   D   S   T   D   T   E   S   N   N   E   E   R   S   A   G   V   S   G  102
IKpcr    acagcagccacaggtggtctcatctacctgaccaaccacatggctccaggtatgagaaat 420
          T   A   A   T   G   G   L   I   Y   L   T   N   H   M   A   P   G   M   R   N  122
IKpcr    ggaggcctgccaggggtaaaggaagaacaacaccggcagtttgaggctttgcgagcagca 480
          G   G   L   P   G   V   K   E   E   Q   H   R   Q   F   E   A   L   R   A   A  142
IKpcr    ggaatggatttgagtatagcgtcatcagaaggatttaaggtgctaagtggagatggagaa 540
          G   M   D   L   S   I   A   S   S   E   G   F   K   V   L   S   G   D   G   E  162
IKpcr    gaattgagcgtaccgctgtatccactgcagagtttgttcctggttcatgtcatgtacac 600
          E   L   S   V   P   L   Y   P   L   Q   S   F   V   P   G   S   C   H   V   H  182
IKpcr    gatccatatgctaagtgagtcgtattacg                                629
          D   P   Y   A                                                186
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hIk    GDKGLSDTPYDSSASYEKENEMMKSHVMDQAINNAINYLGAESLRPLVQTPPGGSEVVPV
cIk    GEKCLSDLPYDATTNYEKENEIMQTHVIDQAINNAISYLGAESLRPLVQTPVGSEVVPV
tIk    GEKRFSNISFEGGPG    ELMQPHVIDQAINSAINYLGAESLRPLIQTSPTSSDM GV
zIk                      SHVMDQAINSAISYLGAESLRPLVQTSPGSADM  V

hIk    ISPMYQLHKPLAEGTPRSNHSAQDSAVENLLLLSKAKLVPSEREASPSNSCQDSTDTESN
cIk    ISPMYQLHKPLGDNQTRSNHTAQDSAVENLLLLSKAKSVSSERDASPSNSCQDSTDTESN
tIk    MGSMYPLHKP    PAEGHGLSAKDSAAENLLLLAKSKSASSEKDGSPSHSGQDSTDTESN
zIk    VSPLYNLHK   SQTAEGNGVSAKDSAAEHLLLLSKSKSASVDKDGSPSPSRQDSTDTESN

hIk    NEEQ        RSGLIYLTNHIAPHARNGL  SLKEE HRAYDLLRAA     SENSQD
cIk    NEE         RSGLIYLTNHIGPHARNGI  SVKEE SRQFDVLRAG     TDNSQD
tIk    NEEK AGV    GASGLIYLTNHITSGVRNGVLPLVKEEQQRQYEAMRAS   IEIAS E
zIk    NEERSAGVSGTAATGGLIYLTNHMAPGMRNGGLPGVKEEQQRHFEALRAAGMDLSIASSE

hIk    ALRVVSTSGEQMKVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSS
cIk    AFKVISSNGEQVRVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSS
tIk    GFKVLSGEGEQVRAYRCEHCRILFLDHVMYTIHMGCHGFRDPFECNLCGHRSQDRYEFSS
zIk    GFKVLSGDGEELsvplyplqsfvpgschvhdpha

hIk    HITRGEHRFHMS
cIk    HITRGEHRFHMS
tIk    HMTRGEHRY
zIk
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Fig. 2. Alignment of IkPCR amino acid sequence (zIk) with *ika1* exon 7 sequences from human (hIk), chicken (cIk), and trout (tIk). The 22 amino acid region of no homology (lower case letters) at the 3' end of the zebrafish sequence is probably an experimental artifact.

lauroyl sarcosine, and .2% SDS. The blot was washed once with .2X SSC/.1% SDS, twice with .1X SSC/.1% SDS, and once with .05X SSC/.1% SDS for 15 minutes each at 65°C.

Sequencing

DNA sequencing was performed using [^{35}S]dATPαS and the Sequenase kit (USBiochemicals, Cleveland, OH). IkPCR was sequenced using primers M13 universal and M13 reverse from Litmus29. Both strands were completely sequenced. Subclones of IK4A were sequenced with EX15 and EX16 from the vector, and internal primers Ik5' (TTGATGCGGTGATCCATCAC) and Ik3' (TCATCTAC-CTGACCAACCAC).

Whole Mount *in situ* Hybridization

Whole mount *in situ* hybridization was performed essentially as described (Willett, *et al.*, 1997 a). Embryos were fixed in 4% paraformaldehyde/PBS (.16 M NaCl, .027 M KCl, .02 M sodium phosphate, pH 7.3) overnight at 4°C and dehydrated in methanol. After stepwise rehydration, they were refixed in 4% PFA for 20 minutes, rinsed with PBST [PBS plus .1% Tween (Sigma, St. Louis, MO)], and prehybridized in 50% formamide, 5X SSC, 50 μg/ml heparin, 500 μg/ml tRNA, .1% Tween-20, and 9.2 μl/ml 1.0 M citric acid (pH 6.0). The embryos were hybridized at 65°C overnight in 5.0 μg/ml probe. Embryos were blocked in 2% newborn calf serum and 2 mg/ml BSA for one hour at room temperature before antibody incubation. Anti-digoxigenin Fab fragments (Boehringer-Mannheim) were diluted 1:400 in PBST and pre-absorbed to the same number and age of embryos as those being stained. The antibody was diluted again to a final dilution of 1:4000 for incubation. The embryos were stained in 3.5 μl 5-bromo-4-chloro-3-indoyl-phosphate and 4.5 μl

4-nitroblue tetrazolium chloride (both from Boehringer-Mannheim) per ml of alkaline phosphatase buffer [100 mM Tris HCl (pH 9.5), 50 mM MgCl2, 100 mM NaCl, .2% Tween-20, .2% Triton-X 100, 1.2 mg/ml fresh Levamisol]. Staining in the ICM was visible after approximately three hours at room temperature. Fish were then refixed in 4% PFA and photographed in 3% methyl cellulose.

Results

Sequencing IkPCR insert

The DNA sequence of the IkPCR insert was determined and translated (Figure 1). The amino acid sequence was compared to exon 7 of human, chicken, and trout Ikaros (Figure 2). This comparison revealed 22 amino acids at the 3' end of IkPCR which show no homology to any of the other sequences. This addition is possibly an artifact of PCR or the cloning process. Other regions of the sequences show extensive homology.

Cloning *ika1*

The IkPCR fragment was used to screen a genomic library. A 17.6 kb clone containing exon seven was identified and mapped by restriction digests (Figure 3). The region corresponding to the probe was identified by Southern blotting of several restriction digests. The orientation of the fragment was determined by PCR with internal primers and vector primers (Figure 3). The insert was then subcloned into pBluescript S/K- as three *Sac*I fragments, and an additional set of *Eco*RI/*Xba*I fragments. Sequencing of the ends of EX15 and EX16 (Figure 3) failed to identify any upstream exons of *ika1*.
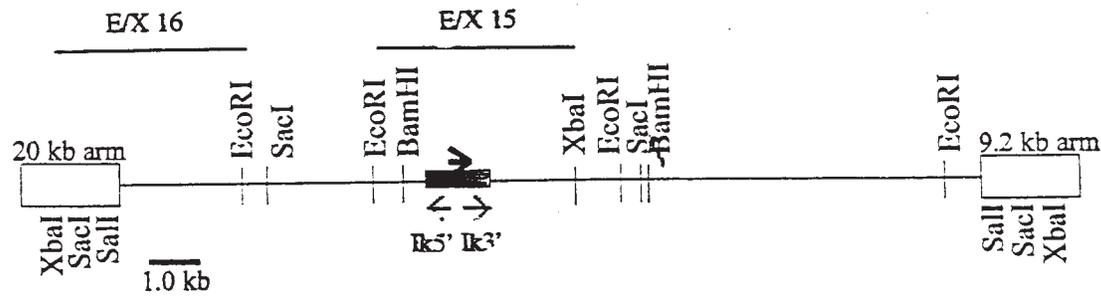
Fig. 3. Restriction map of IK4A, a λ clone of part of the zebrafish *ika1* locus. IkPCR probe location is indicated by the shaded box. Primers Ik5′ and Ik3′ were used to determine the direction of the probe. The ends of EX16 and EX15 *Eco*RI/*Xba*I subclones were also sequenced with primers from the vector.
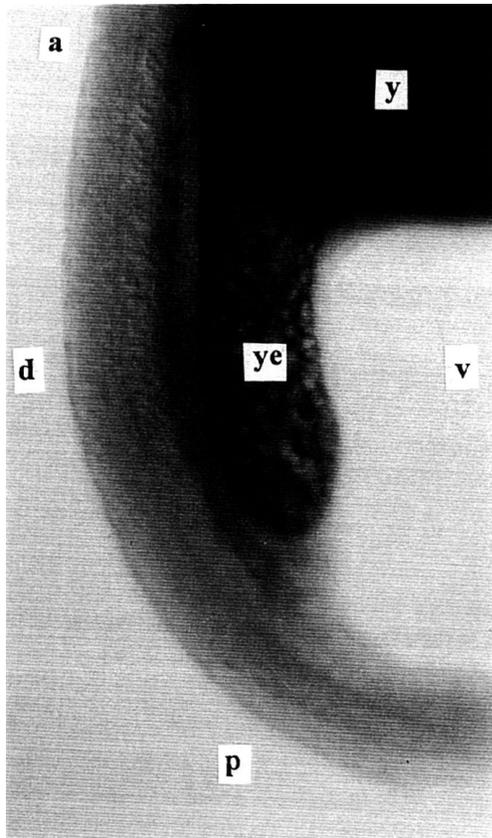
Fig. 4. Whole mount *in situ* hybridization of *ika1* expression at 24 hr. Staining (purple) can clearly be seen along the ICM in the tail. a, anterior; p, posterior; d, dorsal; v, ventral; y, yolk mass; ye, yolk extension.

Expression of *ika1*

Whole mount *in situ* hybridization on 24 hr embryos showed *ika1* expression in the intermediate cell mass (Figure 4) and the head. Previous studies have mapped and identified the ICM as an early site of hematopoiesis (Weinstein *et al.*, 1996). Expression in other ages of fish has not yet been reliably characterized.

Cloning the *rag1* 5′ region

The most 5′ 3.5 kb of the previously obtained *rag* clone (clone 10; Figure 1 of Willett *et al.*, 1997 a) was used as a probe to isolate another clone, designated 10C, containing more insert 5′ of the *rag1* coding region. Restriction mapping is currently being carried out on this clone.

Construction of *rag1* promoter-GFP fusion

Injection of the RGM2 construct yields minimally specific transgene expression in the thymus with significant expression in muscle (S. Lin, personal communication). It is likely that RGM2 is missing crucial regulatory regions of the *rag1* gene. Therefore, we are adding putative regulatory regions to RGM2, including an additional 3.5 kb upstream sequence that is contained on the original λ clone. We are also adding the *rag1/rag2* intergenic region 3′ to GFP. In case this is not sufficient, we will add sequence from the upstream clone, 10C, described above.

Discussion

We have cloned the zebrafish *ika1* gene as a marker for early immune system development. Expression can be detected in a specific pattern at 24 hr by whole mount *in situ* hybridization. We hope to further refine the *in situ* conditions in order to remove background and non-specific staining. We will continue to investigate *ika1* expression in embryos of different ages.

We have shown that *ika1* can be used as a marker for early hematopoietic development. Previously, we showed that *rag1* can be used to study later development. Armed with these assays for different developmental stages, we are now prepared to assay for mutations affecting

hematopoiesis. The cloning of the genes identified in the Driever *et al*. and Haffter *et al*. screen is currently difficult because it relies on positional cloning methods. The lab of Nancy Hopkins at MIT is undertaking a complementary screen which would aid in isolating developmentally important genes. Using virus-mediated insertional mutagenesis, they will screen for mutations up to day five of development. A pilot screen of 217 insertions has already identified three genes and facilitated the cloning of two of them (Gaiano *et al*., 1996; Allende *et al*., 1996). We plan to screen insertionally mutagenized embyros by whole mount *in situ* hybridization with *ika1* at 24 hours and *rag1* at five days.

An additional method to identify mutations in such screens is the use of transgenic fish. Fish with the *ika1* or *rag1* promoter controlling the expression of a GFP reporter gene might facilitate quick and sensitive screening. Such fish have already been created using *gta1* (Long *et al*., 1997) and *gta2* (Meng *et al*., 1997) promoters. In order to make the *rag1* transgenic fish, we have obtained additional *rag1* upstream sequences which can be used to reprobe a genomic library and walk to the promoter. We are also using 5' RACE (Rapid Amplification of cDNA Ends) to reach the promoter regions of both *ika1* and *rag1*. We plan to use the transgenic fish to identify mutants affecting specific pathways. This could be done in separate screens by chemical mutagenesis.

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