Conserved Functions of Ikaros in Vertebrate Lymphocyte Development: Genetic Evidence for Distinct Larval and Adult Phases of T Cell Development and Two Lineages of B Cells in Zebrafish

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*J Immunol* 2006; 177:2463-2476; doi: 10.4049/jimmunol.177.4.2463
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Conserved Functions of Ikaros in Vertebrate Lymphocyte Development: Genetic Evidence for Distinct Larval and Adult Phases of T Cell Development and Two Lineages of B Cells in Zebrafish

Michael Schorpp, Mike Bialecki, Dagmar Diekhoff, Brigitte Walderich, Jörg Odenthal, Hans-Martin Maischein, Agustin G. Zapata, Tübingen 2000 Screen Consortium, Freiburg Screening Group, and Thomas Boehm

Zebrafish has been advocated as an alternative animal model to study lymphocyte development, although the similarities in the genetic requirements of lymphopoiesis between fish and mammals have not yet been investigated. In this study, we examine the role of the transcription factor Ikaros in zebrafish lymphopoiesis. In fish larvae homozygous for an ikaros allele predicted to lack the C-terminal zinc fingers, T lymphopoiesis is absent; the presence of \( \gamma D \mu J \mu \) rearrangements in adolescent fish is delayed in mutants. In adolescent mutant fish, \( T \) cells expressing \( \text{terb} \) and \( \text{terd} \) and \( B \) cells expressing \( \text{igm} \) are formed with low efficiency and display an oligoclonal Ag receptor repertoire. By contrast, \( B \) cells expressing the \( \text{igz} \) isotype do not develop, providing genetic evidence for two separate \( B \) cell lineages in zebrafish. Thus, Ikaros appears to play similar roles in fish and mammalian lymphopoiesis.

\[ \text{The Journal of Immunology, 2006, 177: 2463–2476.} \]

Development of lymphocytes depends on a complex set of transcription factors orchestrating their cell fate specification, commitment, and differentiation. Hemopoietic stem cells give rise to lymphocyte progenitors that diverge to develop into the different subsets of \( T \) and \( B \) cells. Genetic studies using gene targeting and the analysis of tumor-associated lesions generated a detailed picture of gene regulatory networks that govern \( T \) and \( B \) cell specification and differentiation (1, 2). One central component among these transcriptional regulators is the product of the \( \text{Ikaros} \) gene. Ikaros is a key transcription factor whose activating function is required for lymphocyte development at various levels, where it cooperates with additional members of the \( \text{Ikaros} \) gene family and other transcription factors (3). Ikaros associates with chromatin remodeling complexes (4), probably explaining its repressor activity and its function as a tumor suppressor. Originally identified as binding to regulatory regions of the terminal deoxynucleotidyltransferase (5) and the CD3\( \delta \) (6) genes, it was later found to give rise to a complex set of protein isoforms that are generated following alternative splicing of pre-mRNA (3). The Ikaros protein is characterized by the presence of six zinc finger domains. The first four zinc fingers engage in specific DNA binding and are variously contained in the different Ikaros isoforms; the last two fingers are required for protein-protein interactions and engage in homo- and heterodimerizations with Ikaros family members (7). In the mouse, several alleles of the \( \text{Ikaros} \) gene have been generated. The removal of the last exon of the gene (encoding the last two zinc fingers) is considered a null mutation and causes lack of \( B \) and fetal \( T \) cell development (8). A dominant-negatively acting version of Ikaros, generated by removal of the first two zinc fingers additionally inhibits development of adult \( T \) and \( NK \) cell development and leads to tumorigenesis (9). A mouse strain with low levels of the Ikaros protein was recently generated by removal of the second exon of the gene; this mutant protein retains the zinc fingers of the wild-type protein and supports residual \( B \) cell development reminiscent of a hypomorph (10). A single base substitution disrupting the third zinc finger of the Ikaros protein was recently identified from a library of ethyl-nitrosourea (ENU) mutagenized mice; this led to widespread failure of hemolymphoid differentiation (11) due to the formation of nonfunctional protein complexes.

Homologs of the Ikaros transcription factor gene have been identified in the genome of all vertebrates studied to date, including zebrafish (12). It is unknown, however, whether this structural conservation also extends to evolutionarily conserved functions. The zebrafish has been recently advocated as a suitable model to investigate the genetic basis of lymphocyte and lymphoid organ development (13–15), but information about these processes in the fish is still scarce. Genetic analysis of zebrafish development has

\[ \text{7 Abbreviations used in this paper: ENU, ethyl-nitrosourea; hpf, hours postfertilization; WGS, whole genome sequence; dpf, days postfertilization; BAC, bacterial artificial chromosome; WISH, whole mount in situ hybridization.} \]

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### Table I. Oligonucleotide primers used in this study

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<tr>
<th>Gene/Fragment</th>
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<tr>
<td><em>ikaros</em>001*</td>
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<td><em>ikaros</em>002*</td>
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<td>cDNA amplification</td>
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<td>aagaattcgaagcccatatgcg</td>
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<td>tcRCb2 (first round)</td>
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<td>530</td>
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<tr>
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<tr>
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<td>aagaattcgaagcccatatgcg</td>
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<td>Ig (VDIC) complete transcripts</td>
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<td>Ig VDJ CDNA/genomic DNA amplification</td>
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<td>eFla</td>
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<td>Ig VJ3-J (first round)</td>
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<td>gtaaattcgaagcccatatgcg</td>
<td>aagaattcgaagcccatatgcg</td>
<td>450</td>
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* (TAG)n simple repeat in intron 3.
* Detection of 249980 mutation (loss of Tsp45I site) in exon 9 in genomic DNA.
* Detection of 249980 mutation (loss of Tsp45I site) in exon 9 in cDNA.
* Vb1.5, Vb12, Vb14.5, and Vb17.5 are Vb elements present in sequence CAAA01000103.
* Taken from Ref. 35.
become possible through the application of forward genetic screens following ENU (16, 17) or insertional (18) mutagenesis. To develop the zebrafish model as a useful addition to the tool box of immunological research, it is important to establish not only the extent of similarities but also the degree of uniqueness of its immune system compared with the much better-studied mammalian model systems. Given the central role that Ikaros plays in lymphocyte development in mammals, we considered it worthwhile to establish the function of Ikaros in zebrafish lymphopoiesis. To this end, we made use of our collection of zebrafish mutant lines that were derived from a previously described pilot screen (19) and the recent Tübingen 2000 screen (M. Schropp et al., manuscript in preparation).

In this study, we describe the phenotype of zebrafish homozygous for a recessive ikaros allele containing a nonsense mutation in the last coding exon, similar to the situation in a loss-of-function mutation in the mouse. Our results define larval and adult stages of lymphopoiesis in the zebrafish, provide genetic evidence for two different B cell lineages, and reveal remarkable similarities of fish and mammalian lymphocyte development.

Materials and Methods
ENU mutagenesis, inbreeding schedule, mutant recovery, classification, and complementation testing

Adult zebrafish males of the Tübingen line were incubated in buffered E3 medium containing ENU as previously reported (16). After mutagenesis, males were repeatedly mated to normal females to generate over 10,000 F1 heterozygous offspring. To confirm linkage to the ENU gene, an intragenic marker was used.

Twenty to 30 larvae of each F1 clutch were screened via rag1 in situ hybridization. Based on the results on a small-scale pilot screen (19), we used the extent and pattern of rag1 staining in larvae at 120 h postfertilization (hpf) as a suitable marker to detect alterations in thymus development. At this time point in larval development, rag1 staining is confined to the thymus rudiment (19, 20). rag1 staining was expected to be reduced or absent in case of disturbed lymphoid development and also in case of aberrant development of the thymic stromal microenvironment and secondary impairment of thymopoiesis. A clutch was considered positive when 20–30% of the larvae showed a similar alteration in the rag1 expression pattern or intensity. To confirm and recover mutations, F2 pairs producing putative mutants among their F3 offspring were cross-bred, and the resulting new F3 families underwent the same inbreeding and screening procedure.

To determine whether two mutations causing similar phenotypes reside in the same or in two different genes, complementation analyses were performed, crossing a heterozygous fish of one mutation with a heterozygous fish of the other mutation. Alleric mutations fail to complement each other in transheterozygous embryos, which show the mutant phenotype like homozygotes of either allele. If the mutations are in different genes, the double heterozygous offspring show a wild-type phenotype.

A total of 141 mutants were detected in the primary analysis; of these, 92 were not yet covered for in-depth analysis. The remaining 49 mutants were initially classified based on the results of rag1 in situ hybridization and gross morphology of fish. The first group consists of mutants that do not exhibit any obvious abnormality other than lack of or reduced rag1 staining. The second group of fish additionally displays developmental abnormalities, some with craniofacial defects of varying degrees.

Using molecular probes, all mutants were subsequently analyzed for potential abnormalities of hemopoietic cells; development of pharyngeal endoderm and ectoderm, and structures derived from neural crest at various time points during the first 5 days of embryonic development. Differentiation of hemopoietic cells in the intermediate cell mass, a site of embryonic blood formation, was assessed by staining with scf, a gene that specifies hemopoietic and vascular progenitor cells (21), gata1, a gene required for red cell development (22), and ikaros, as a putative marker of lymphoid progenitors in zebrafish (12). The arrival and early differentiation of T cell progenitors in the thymic rudiment was assessed by staining with ikaros, ccr9, the zebrafish homolog of the mammalian chemokine receptor 9, a marker of early T cells in the mouse (23, 24), t-plastin, a marker of the myeloid lineage (25), rag1, a marker of immature lymphoid cells rearranging their Ag receptor loci (19, 20, 26), TCR β (tcfrβ), and δ (tcfrδ) (see Results), as markers for αβ and γδ T cells, respectively. Development of the pharyngeal arches was analyzed using gem2, the zebrafish homolog of the mouse Gcm2 gene as a maker for pharyngeal ectoderm (27), and foxl1 (28), the zebrafish homolog of the mouse Foxl1 gene that is required for differentiation of thymic epithelial cells (29) and expressed in endodermal derivatives. Neural crest development was assessed by dlx2 expression (30) and cartilage formation by Alcian blue stainings.

Genomic localization of zebrafish mutations was determined using the Tübingen marker set for genome scans (version 4) on F2 Tübingen × Wik crosses of the mutant carriers. Primer sequences are available from the MGH website (http://zebrafish.mgh.harvard.edu). The ikaros gene, which is in the HS mapping panel has been linked to 9c359; in the MGH mapping panel, z5359 and z10070 map to linkage group 13 at 40 cM; cf ZFIN database at (http://zfin.org/cgi-bin/webdriver?MIval=aa-newmrkselect.apg). To confirm linkage to the ikaros gene, an intragenic marker was used (Table I).

In situ hybridization and electron microscopic analysis

For initial screening, F3 clutches were incubated in the presence of 0.25 mM 1-phenyl-2-thiourea (Sigma-Aldrich) to inhibit melanin synthesis, and fixed in 4% paraformaldehyde/PBS at 120 hpf. Whole mount in situ hybridization (WHO) was conducted in specially designed 48-well plates (Aldinger) with digoxigenin-labeled probes for growth hormone (31), and rag1, a marker for thymic T cells (20, 26), following standard protocols (19). Details for these and all other probes used in this study can be found in Table II. Analysis by electron microscopy followed earlier procedures (20).

Rearrangement, expression, TUNEL, and proliferation assays

To analyze igμ rearrangements in genomic DNA, a published protocol was used (32); igμ rearrangement assays used primers listed in Table I. VDJC containing cDNAs from igμ, igλ, tcfrβ, tcfrδ genes were amplified using primers listed in Table I. Fragments were cloned and sequenced. TUNEL assays were performed using Roche in situ cell death detection kit; the wound healing and proliferation assays were performed by incubation with BrdU at a concentration of 150 μg/ml in fish water and the incorporation was assessed using the Roche BrdU Labeling and Detection kit II. All analyses were performed for at least two animals of each genotype; since no significant interindividual differences were found, the results per genotype were pooled.

Data mining

The zebrafish ccr9 gene was identified using the human CCR9 coding sequence accession NM_031200 as a query sequence against the zebrafish whole genome sequence (WGS) trace archive using the mega-blast algorithm. Using these initial genomic sequences that covered exon 3 of zebrafish ccr9, RT-PCR primers were designed for 5′-RACE to obtain cDNA sequence information (data not shown). This resulted in ~150 bp of sequence information (data not shown).

Table II. Gene-specific in situ hybridization probes used in this study

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<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Nucleotides to X</th>
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<td>foxn1</td>
<td>NM_212573</td>
<td>395–1490</td>
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<tr>
<td>gcm2</td>
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<td>399844–410396</td>
</tr>
<tr>
<td>gata1</td>
<td>NM_131234</td>
<td>1–1694</td>
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<td>scf</td>
<td>AF038873</td>
<td>1–2527</td>
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<td>AF157110</td>
<td>3–766</td>
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<td>U71093</td>
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<td>ikaros</td>
<td>AF092175</td>
<td>1–2309</td>
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<td>This study</td>
<td>785–1341 in Fig. 3</td>
</tr>
<tr>
<td>tcfrb-c</td>
<td>This study</td>
<td>Italics in Fig. 8</td>
</tr>
<tr>
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<td>pcnA</td>
<td>NM_131404</td>
<td>1–1137</td>
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*Reverse complement of AL596139; corresponds to the tcdr cDNA sequence encoded in three exons; see Fig. 7.*
additional sequence at the 5'-end, which is contained in two exons, as determined by blastn comparisons against the WGS trace archive. The initiation codon is found in exon 1. The location of the poly(A) site has not been determined.

The tcrβ C region gene was identified using the flounder tcrδ C region domain (nt 18922–19064 and 19137–19376 in accession number AL596139) as a query sequence against the zebrafish WGS trace archive using the blastn function of the megablast algorithm. Using these initial genomic sequences, a sequenced bacterial artificial chromosome (BAC) clone, assigned to linkage group 2, was identified that contained initial genomic sequences, a sequenced bacterial artificial chromosome AL596139) as a query sequence against the zebrafish WGS trace archive. The relevant Cβ elements downstream of Cα (accession number BX681417.10). RT-PCR primers were designed to link the first and last Cα exons and revealed an additional exon not predicted by gene structure algorithms. The BAC sequence does not contain V elements for ~190 kb upstream of D61, indicating that Va/Vδ elements may be in inverted configuration downstream of Ca as observed in Tetraodon nigroviridis (33) and other teleosts. Indeed, a contig of three BACs joins Ja, Ca, and Vδ/Vα elements, supporting this assumption. So far, a total of five V sequences (contained in DD332, B14, B22, B35, B38 cDNA clones) were identified spliced to Cα sequences (data not shown for B14, B22, B38; for B35, see Fig. 7; the VδDD332 element is contained in trace sequence zfish44910-13e08.p1k and BAC clone BUMSI-257N2, accession number AL928815.15). These V element sequences (with the exception of VδDD332) have previously been annotated as Vα sequences (the sequences for B14 and B22 were too short to allow an unambiguous assignment to a specific Vα element); B38 has been annotated as VαL4 (cf accession AL9591674); B35 has been annotated as VαL3-36 (cf accession AL952550.11). The tcrβ C region genes were identified using the various tcrδ cDNA or expressed sequence tag sequences derived from other teleost fish sequences as query sequences against the zebrafish WGS trace archive using the blastn function of the megablast algorithm. These initial genomic sequences were assembled into contigs and manually curated.

FIGURE 1. A nonsense mutation in the last exon of the ikaros gene of line IT325 causes abnormal larval thymopoiesis. A. Sequence comparison of C-terminal exon-encoded Ikars proteins from mouse (top line; red letters) and zebrafish (bottom; identities to mouse are also in red). The position of the stop codon in IT325 is marked by an asterisk (*); the diagnostic residues of the two C2H2-zinc fingers are highlighted. The mouse bipartite activation domain at the beginning of exon 7 sequences is highlighted in blue. The relevant C>T transition in genomic DNA of IT325 mutants occurs at position 61033 in the genomic sequence (reverse complement of GenBank accession number BX890571.8) and at position 1166 in the cDNA sequence (GenBank accession number AF092175). B, WISH analysis (rag1, left panels; ikaros, right panel) of wild-type fish and mutants homozygous for the ikaros mutants allele at the indicated time points after fertilization. The position of one of the two thymic rudiments is circled for orientation. In the rag1 assays, a probe specific for growth hormone was included as an internal control of the hybridization process; it stains the hypophysis that is located medially behind the developing eyes (arrowheads). The ikaros probe not only stains lymphocytes but also certain structures in the eyes and the developing CNS; most of these sites of hybridization are out of the focal plane in these microphotographs.

Table III. PhenoType of ikaros morphants

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<tr>
<th>Treatmenta</th>
<th>Number of rag1-positive cells</th>
<th>Number of embryos</th>
<th>Difference</th>
<th>Σ</th>
<th>p*b</th>
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<td>0</td>
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<td>61</td>
<td>82</td>
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<tr>
<td>Control</td>
<td>0</td>
<td>1</td>
<td>12</td>
<td>72</td>
<td>74</td>
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</tbody>
</table>

*a Wild-type zebrafish were injected with 1 pl of 200 μM ikaros-specific antisense morpholinos (I: 5'-ttctgctgcctctcactcct; II: 5'-ctctgagctctct-tgttct) at the one-cell stage. Four days later, embryos were fixed and whole-mount in situ hybridization was performed with a rag1 probe, and the number of rag1-positive cells counted in the thymic primordium. The in situ hybridization procedure was controlled by use of an internal standard probe, growth hormone, which also controls for developmental abnormalities. A control morpholino (5'-tccgtggagctgagacgacttcgac) was used to assess nonspecific effects of injection.

*b Fisher’s exact probability test, two-tailed: control vs antisense morpholinos; Bonferroni correction for multiple testing.
for consistency; the assembly process yielded a single contig for Cβ1 (with some uncertainty because of a tetranucleotide repeat region between exons 3 and 4), and two contigs for Cβ2. Some of these sequences are contained in the sequence of contig assembly CAA01000103, which also contains Vβ elements. The assembled sequence of CAA01000103 (version of January 19, 2005) awaits expert curation and is probably not correct, because some sequences of C region genes are in incorrect order possibly due to many repeats found in this sequence. Upstream of Cβ1, one D element (Dβ1.1) and several J elements (Jβ1.1 to Jβ1.23) were identified (see Table IV). Five further putative Jβ1 elements were found in cDNAs and confirmed by the presence of equivalent genomic sequences in WGS trace archive; a further three Jβ1 elements were detected in cDNAs but not in genomic sequences. Upstream of Cβ2, no D, but a single J element (Jβ2.1; see Table IV) was identified. One other Jβ element (possibly also belonging to the Jβ2 cluster as judged from the sequence of cDNAs containing this element) was identified in assembly sequence CAA01003749. This arrangement of Jβ elements is reflected in cDNA sequences that generally have the structure V-D1-J1-C1 and V-J2-C2 (data not shown).

Results

Identification of a nonsense mutation in the ikaros gene

The phenotype of mice with a truncated Ikaros gene indicates that the null phenotype of this gene is characterized by the complete lack of fetal thymopoiesis. In our collection of ENU-generated zebrafish mutants, several lines exhibited a complete lack of thymopoiesis as determined by WISH with rag1-specific probes in the absence of other developmental defects at 5 days postfertilization (dpf). In zebrafish, the larval stage is commonly considered to last for the first 2 wk, followed by an adolescent period up to 3 mo of age. To identify potential ikaros mutants among this subgroup, we tested for genetic linkage of mutations to chromosome (linkage group) 13, to which ikaros has previously been assigned. Segregation analysis using a panel of simple sequence length polymorphism markers spanning the entire zebrafish genome localized the mutation in line IT325 between markers z5395 and z13250 and very close to marker z10070 (zero recombination events in 168 meioses initially analyzed). Because of the close genetic linkage between the ikaros locus and the map position of the mutation in IT325, the ikaros gene was sequenced in this line. We identified a nonsense mutation at the beginning of the last coding exon of the ikaros gene (allele designation t24980). This exon is equivalent to exon 7 in the mouse Ikaros gene. A C>T transition converts a CAA (Q360) codon to a TAA (termination) codon. The predicted truncated Ikaros protein encoded by the t24980 allele is 177 aa shorter than the wild-type protein, leaving intact the putative bipartite activation domain, but removing the two C-terminal zinc fingers implicated in essential protein-protein interactions (7) (Fig. 1A). To ascertain that the phenotype observed in IT325 mutants indeed segregated with the identified ikaros mutation, we developed an intragenic polymorphic marker (ikaros001, located in the third intron of the ikaros gene; see Table I). It showed tight genetic linkage (zero recombinants in 1020 meioses) and all mutants analyzed were homozygous for the nonsense mutation. This strongly suggests that the phenotype in IT325 mutants is caused by the mutation in the ikaros gene. This conclusion was supported by the finding of reduced numbers of rag1-positive lymphocytes in the thymus at 4 dpf in ikaros morphants (Table III). Collectively, these experiments indicated that the phenotype in IT325 mutants was caused by the nonsense mutation in the ikaros gene.

Characterization of fish homozygous for the ikaros(t24980) allele

Fish homozygous for the t24980 allele lacked rag1 and ikaros expression in the thymus up to the first 8 dpf as determined by WISH (Fig. 1B). Likewise, ccr9 expression in the thymic rudiment (Fig. 2A), which in the mouse is a marker of early T cell progenitors (23) was absent (see Fig. 3 for characterization of the zebrafish ccr9 gene, and Table II for information about all other probes used for in situ hybridization). The apparent lack of lymphocyte progenitors was confirmed by WISH with probes specific for the C regions of the tcrB2 and tcrG6 genes (Fig. 4A), and RT-PCR analyses for these genes (data not shown). A general hemopoietic defect in ikaros mutants was ruled out by the normal expression patterns of gata1 and scl (Fig. 2, B and C) at 24 hpf. Furthermore, the thymic microenvironment was not disturbed in the mutants as shown by normal expression patterns for gcm2, foxn1 (Fig. 2A), and dlx2 and the normal appearance of cartilage in branchial arches (Fig. 2D). The foxn1 hybridization pattern in t24980 mutants (Fig. 2A) was indicative of a compact arrangement of epithelial cells, presumably because no hemopoietic progenitors were present in the thymic rudiment that disperse the resident epithelial cells. This finding was supported by the results of electron microscopy (Fig. 2, A and D), which was consistent with the absence of normal lymphocytes at this time point (31).

FIGURE 2. Characterization of ikaros(t24980) mutants using whole mount RNA in situ hybridization at various time points after fertilization. A, Larvae at 3 dpf (gcm2) and 5 dpf (ccr9, foxn1). The pole of one of the two thymic rudiments is circled for orientation. B, Larvae at 24 hpf (scl, gata1, l-plastin (left panel); note the presence of macrophages in both wild type and mutants (arrowheads). The right panel in l-plastin stains is from 5 dpf, indicating staining in the thymus only for wild type. C, High-power views of the intermediate cell mass at 24 hpf. D, Thirty-two hours postfertilization larvae (dlx2) and 5 dpf (Alican blue staining; visualization of branchial cartilage).
microscopic studies. The wild-type thymus was covered by pharyngeal epithelium and contains numerous lymphoblasts and lymphocytes among thymic epithelial cells; in the mutant thymus, the lymphoid cells were completely missing, whereas the epithelial structures appeared normal (Fig. 4B). Collectively, these studies are compatible with the notion that the t24980 allele affects T lymphocytic differentiation at some prethymic stage. Heterozygous animals analyzed according to the above criteria were completely normal up to 8 dpf, suggesting the recessive nature of this allele.

TCR /H9252 and /H9254 loci in zebrafish

The analysis of zebrafish lymphopoiesis is complicated by the fact that no cell surface markers are available that facilitate such analyses in mouse and human systems. Although a recently developed transgenic zebrafish line facilitates gross analyses of the entire T lineages (34), it does not allow the detailed analysis of B and T cell subsets required for the present experiments. Therefore, we have developed specific reagents to examine /H9252 and /H9254 expressing cells by in situ hybridization and sequence analysis of their Ag receptor gene rearrangements.

Using a combination of database mining and cDNA cloning (see Materials and Methods and Figs. 5–7), an initial characterization of zebrafish /H9252 and /H9254 loci was conducted. The /H9254 C region gene of zebrafish was identified using the flounder /H9254 C region domain as a query sequence against the zebrafish WGS trace archive. Using these initial genomic sequences, a sequenced BAC clone was identified that contained three /H9254, two /H9254, the three /H9254 C region exons, and numerous presumptive J/H9254 elements downstream of C/H9254 (Figs. 5 and 6). Some V sequences identified in /H9254

cDNAs were also found in tcrα cDNAs, indicating that tcrδ and tcrα shared the same V elements (data not shown). Because Vα/Vδ and Jα/Cα elements were linked to each other in inverted orientation, Vα/Vδ elements may be located, in an inverted configuration, downstream of Cα as observed in T. nigroviridis (33) and other teleosts.

Analysis of expressed sequence tag sequences revealed two different C regions with significant homology to tcrβ genes, and several putative Vβ elements. These cDNA sequences were localized to several partially sequenced BAC clones; further analysis suggested that the two tcrβ regions are tandemly arranged, with a single D element (designated Dβ1.1) associated with the first complex. Upstream of the four Cβ1 exons, >20 Jβ1 elements were identified in the available (incomplete) contig sequence; further Jβ1 elements were identified in the cDNA clones investigated in this study (Table IV). The cDNAs containing the Cβ2 gene lacked readily identifiable D sequences and contained either of two Jβ elements (Jβ2.1 and Jβ2.2). In contrast to Cβ1, the C region of tcrβ2 was found to be encoded in three exons. During ontogeny, complete (VJC) tcrβ transcripts were first detected with Cβ2 sequences (beginning 4 dpf), whereasVDJC transcripts with Cβ1 sequences were not detected up to 7 dpf. In adult tissues, both forms were coexpressed. Complete VDJC tcrδ transcripts appeared at 5 dpf. Incomplete (sterile) transcripts lacking V elements were found considerably earlier (tcrβ2 and tcrδ from 2 dpf onward; data not shown).

Collectively, these studies provided specific reagents to examine tcrβ and tcrδ expressing cells by in situ hybridization and sequence analysis of VDJ rearrangements.

**T cell development in ikaros<sup>24980</sup> homozygous mutants**

Interestingly, homozygous mutants survived for >17 mo (the latest time point of analysis) under nonsterile conditions and were fertile. Because it appeared unlikely that lymphopenic fish would survive for such long periods, we examined the possibility that lymphocyte development recovered at later stages of development in ikaros mutants. To this end, we examined lymphocyte development in the thymus by in situ hybridization on tissue sections. Early larval stages of wild-type fish showed an intense staining with the rag1 probe, while mutants lacked evidence for thymopoiesis by this criterion (Figs. 1 and 4). By contrast, adolescent mutant thymus, albeit smaller, contained a large number of rag1-positive cells as well as tcrβ- and tcrδ-expressing cells (Fig. 8A). The first few rag1-positive cells were detected in the thymus of ikaros mutants at the beginning of adolescence, at 14 dpf (data not shown). Electron microscopic studies confirmed the overall similarity of thymic structures between wild-type and mutant fish at these stages (data not shown). This pattern was maintained until 12 mo of age and beyond (data not shown). This indicated that in contrast to adolescent and adult T cell development, larval T cell development was dependent on ikaros function, as previously observed in mice.

We then examined T cell repertoires in the thymus of 3-mo-old adolescent wild-type and mutant fish, respectively. For this purpose, we used sequence analysis of tcrβ1 and tcrδ cDNAs. The majority of VDJC transcripts of tcrβ1 and tcrδ genes amplified from wild-type and mutant fish emanated from productive rearrangements (Fig. 8, B and D). The proportion of unique tcrβ1 sequences among the pool of sequenced cDNAs was significantly higher in wild-type as compared with mutant fish; the same was true for tcrδ cDNAs (Fig. 8, C and E). We noted, however, that the tcrβ1 repertoire was more severely restricted than that of tcrδ clones (compare Fig. 8, C and E; χ<sup>2</sup> = 6.31; p = 0.012). To explore further possible abnormalities of T cell repertoire formation, we examined the pattern of Vδ and Jδ usage (because of the many available Jβ1 elements, a similar analysis for tcrβ1 rearrangements was hampered by insufficient statistical power). Whereas in the wild-type, Jβ1 was used 16 times and Jβ2 17 times all with the Vδ35 element, ikaros mutants used Jβ1 only 4 and Jβ2 29 times (χ<sup>2</sup> = 8.88; p = 0.0003), and the Vδ35 element only 23
FIGURE 6. Characterization of the zebrafish tcr locus. A, Genomic structure. A partial sequence of the tcr locus situated upstream of the \(J_\text{H9254}\) cluster is shown. The first nucleotide shown is nt 193661 in the reverse complement of the BAC sequence (GenBank accession number BX681417.10); the sizes of gaps in the sequence shown are indicated in brackets. The derived protein sequences of \(J_\text{H9254}\) and \(C_\text{H9254}\) exons are indicated in single-letter code; the predicted transmembrane region containing the characteristic positively charged residues is underlined; conserved amino acid residues found in all tcrd chains are in bold; the termination codon is indicated by an asterisk. Splice sites are underlined. The presumptive recombination signal sequences of \(D_\text{H9254}\) and \(J_\text{H9254}\) elements are highlighted in bold, as is the presumptive polyadenylation signal sequence. The most upstream \(J_\text{H9251}\) occurs at position 2470.

B

C

D
of 34 times (the remainder using Vβ35a, see Fig. 6) (χ² = 12.77; p = 0.0004; data not shown). This clearly showed that T cell selection in the thymus was abnormal in 

ikaros mutants.

To explore the biological relevance of these findings further, we repeated the analysis with cDNAs obtained from kidney (an important primary and secondary lymphoid tissue in teleosts) of adult fish (9 mo or older). The results indicated that the severe restriction of the 

tcr-β repertoire persisted in the mutants (Fig. 8 C), whereas the repertoire of 

tcr-β clones had become indistinguishable between wild type and mutant (Fig. 8 E). These results indicated that in the absence of normal 

ikaros function, development of 

tcr-β-expressing T cells was more severely disturbed than that of 

tcr-β-expressing T cells. In both wild-type and 

ikaros mutants, Vβ35 and Jβ2 elements dominated in the sequenced clones, indicating that the skewed T cell repertoire in the thymus was normalized in peripheral tissues. It also suggested that, at least for 

tcr-β-expressing T cells in the periphery, abnormal proliferation did not occur. Collectively, our analyses indicate that thymopoiesis occurs with reduced efficiency in mutant fish with only few cells completing T cell maturation, followed by a certain degree of homeostatic proliferation in the periphery.

Two B cell lineages in zebrafish

Recent reports have identified a second Ig H chain isotype, igh, in zebrafish (35) and rainbow trout (36). It has been proposed that it may be expressed in a separate lineage of B cells (36). Indeed, the structure of the 

igh locus (35) (Fig. 9 A) lends itself to a genetically programmed lineage choice; rearrangement of a VH segment to 

203873 (first nucleotide of Jβ2 coding sequence), −4 kb downstream of the presumptive poly(A) site of Cβ1. B, Nucleotide sequence and derived protein sequence of cDNA clone B35 (Vd35) isolated from 8-wk-old thymus. The position of the intron (i) in the Vβ element is indicated; the nucleotide sequence shown in bold is composed of three joined Dβ elements. C, Nucleotide sequence and derived protein sequence of clone Vd35a isolated from 8-wk-old thymus. The genomic sequence derived from trace sequence zfhsb4910-13e08.p1k is shown and the heptamer/nonamer signal sequences are indicated in bold. D, Protein alignment of the zebrafish 

tcr-β region and the human TCRD (GenBank accession number A35591) sequence. Identical residues are in bold; note the highly conserved transmembrane region.
Table IV. *J̦B* sequences

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| /H9252

* Derived protein sequences are in uppercase letters. Bold letters represent heptamer/nonamer signal sequences. Underlined portions represent splice donor sites.
DμJμ elements deletes the intervening DζJζ elements, much like the situation of the tandemly arranged tcro/ikaros loci whose rearrangement patterns guide the development of αβ and γδ T cells, respectively.

We first examined expression of igμ and igζ in wild-type and ikaros mutants at ≥7 mo. Using RT-PCR, we failed to amplify V_{H}D_JζCζ cDNAs in mutant fish, using primers specific for all V_{H} families (37) tested, whereas V_{H}DμJμCμ cDNAs were readily amplified in wild-type and mutants (Fig. 9B). Surprisingly, V_{H}D_Jζ rearrangements could not be amplified even from total genomic DNA of kidney marrow in ikaros mutants; again, V_{H}DμJμ rearrangements were readily amplified (Fig. 9B). This indicated that igζ-using B cells were absent in ikaros mutant fish and suggested that igζ-expressing B cells required ikaros function for development and/or maintenance; furthermore, the absence of rearranged igζ alleles indicates that the choice of differentiating along the igμ or igζ pathways is not stochastic.

Next, we determined the presence of V_{H}DμJμ- and V_{H}DζJζ-rearrangements in wild-type and mutant fish at earlier stages of development. In wild-type fish, V_{H}DμJμ rearrangements were first detected 3 wk postfertilization, whereas V_{H}D_Jζ rearrangements were first detected 1 wk later. In ikaros mutants, V_{H}DμJμ rearrangements were first detected 4 wk postfertilization, albeit in fewer copies per embryo than in wild types (data not shown). V_{H}D_Jζ rearrangements were never observed in mutant fish. Collectively, these studies indicated that the development of B cells using igμ is delayed but not completely abrogated in ikaros mutants.

Abnormal development of igμ-expressing B cells in ikaros homozygous mutants

B cells represent a large part of lymphoid cells in the head kidney, the fish equivalent of the mammalian bone marrow. igμ cDNA sequences can be readily amplified from both wild-type and mutant tissues using primers specific for all V_{H} families tested (37). For in-depth sequence analysis in our mutants, we arbitrarily chose cDNAs containing V_{H}1 family genes. Analysis of other V_{H} families revealed qualitatively similar results (data not shown). Most igμ cDNA sequences obtained by RT-PCR using V_{H}1- and Cμ-specific primers, respectively, from wild-type and mutant tissues were in-frame (productive) across the V-D-J-C junctions (Fig. 9C). By contrast, the fraction of productively rearranged igμ alleles amplified from genomic DNA was lower (Fig. 9C), suggesting the presence of nonsense-mediated decay of mRNAs with premature termination codons in B cells of zebrafish. A second observation was that the fraction of productively rearranged alleles in genomic DNA was significantly lower in mutant tissues (0.15 in mutant vs 0.75 in wild type). The overrepresentation of nonproductive rearrangements in mutant tissue (χ^2 = 64.75; p < 0.0001) could only be explained by the presence of cells with μ^−/− or μ^−/+ genotypes, because, in the presence of allelic exclusion, igμ^+ cells can only be μ^+/+ or μ^+/− (μ^+); no rearrangement at igμ;
The results showed a slight (25%) increase in head kidney tissues was determined by TUNEL staining at 8 wk postfertilization. Although in these experiments we were unable to differentiate among hemopoietic cells of different cell lineages, the results nevertheless excluded some general defect in apoptosis pathways as a result of loss of normal ikaros function. Next, we examined the general proliferation propensity of hemopoietic tissues in the head kidney of wild-type and ikaros mutants at 8 wk postfertilization. Proliferation was analyzed in two different ways. First, we examined the expression of pcna on sections of head kidney by RNA in situ hybridization to measure the number of proliferating cells. Mutant tissues showed a slight (20%) reduction of labeled cells was observed in mutant tissues, indicating that mutant cells did not proliferate faster than normal ones (data not shown). In a second experiment, 7-wk-old fish were exposed to BrdU in their tank water, the tissues were fixed, and sections were developed for BrdU staining. The results after 5 days of BrdU treatment indicated that the number of BrdU-positive cells (both high and low intensity staining) accumulating during this time period is about the same for wild-type and mutant tissues (data not shown). When fish were exposed to BrdU for 5 days, and then kept for an additional 2 days in the absence of BrdU before analysis, the number of strongly stained nuclei was reduced compared with samples without chase. However, there was no difference in the number of BrdU-positive cells between wild-type and mutant tissues, indicating that mutant cells did not proliferate faster than normal ones (data not shown). Finally, we repeated the BrdU-labeling experiment (10 day labeling period) with 7-mo-old fish. Here, a slight (20%) reduction of labeled cells was observed in mutants (p = 0.01, data not shown), suggesting that overall capacity of proliferation in hemopoietic tissues was somewhat reduced at this age. Collectively, however, our data excluded major changes in apoptosis and proliferation in the absence of normal ikaros function.

We next examined the repertoire of igμ sequences represented in productively and nonproductively rearranged igμ alleles. As shown in Fig. 10, A and B, the sequence diversities differed significantly between wild-type and mutant fish, both for productively and nonproductively rearranged alleles. However, because productively rearranged alleles dominate in the wild-type (Fig. 9C), nonproductively rearranged alleles can be considered passenger sequences in cells with a μ−/− genomic configuration at the igh locus (Fig. 9D). In mutant cells, productively rearranged alleles were the minority and hence did not contribute significantly to the properties of the entire cell population (Fig. 9C). Therefore, with regard to sequence diversity, the most informative comparison was a between the sequence diversities of the dominant types of alleles, the productively rearranged wild-type and nonproductively mutant alleles. The proportion of unique sequences was significantly smaller in nonproductively rearranged alleles of mutant fish (χ² = 18.81; p < 0.0001). A qualitatively similar result was observed for sequences obtained from cDNA, although the low numbers obtained from mutant tissues reduced the power of the statistical analysis. This result strongly suggested the presence of oligoclonal immature igμ− B cell populations in the mutant kidney tissue that outnumbered igμ+ cells. To verify these predictions, we directly examined the presence of igμ+ cells in wild-type and mutant tissues by in situ hybridization and determined the number of total B cells by use of pax5 expression, as a marker of B lineage identity (38). In wild-type tissue, igμ+ and pax5+ cells were present in comparable numbers; in mutant tissue, the ratio of igμ+ to pax5+ cells was much lower, suggesting that immature igμ− cells predominate among pax5+B cells (Fig. 10C).

Next, we examined the cellular composition in the head kidneys of wild-type and mutant fish by FACS analysis. The number of cells with the characteristics of small lymphocytes (39) was significantly reduced in mutant tissue (Fig. 10D). These findings support the notion of abnormal and inefficient lymphoid development in ikaros mutants.

**Discussion**

Zebrafish has only recently been considered as a potential model organism to genetically address immunological problems, such as lymphoid organ and lymphocyte development. At present, knowledge about the immune system of zebrafish is limited (13–15).
Therefore, we focused our studies on the Ikaros transcription factor as a central regulator of lymphopoiesis (3).

The mutation we describe here is similar but not identical to that predicted for the null allele in mouse (8); the mouse allele was generated by deletion of the entire C-terminal exon that contains a bipartite activation domain and two zinc fingers required for protein-protein interactions (8). The *ikaros* allele described here differs from this mouse mutant in that the activation domain is retained in the zebrafish, while the two zinc fingers are also missing. At present, we have no evidence indicating that this allele acts in a dominant-negative fashion, although more detailed analyses are required to firmly establish this conclusion.

Collectively, our results suggest that the tcrβ and tcrδ loci as a tool to assess the development of these two major T cell lineages. Larval development of tcrβ- and tcrδ-expressing T cells is absolutely dependent on normal *ikaros* function. Interestingly, thymopoiesis resumes at later stages (beginning in adolescence), although the T cell repertoire in the thymus of *ikaros* mutants is abnormal and less diverse than in wild-type siblings. A qualitatively similar observation has also been made in mice with an *Ikaros* null mutation, where some subsets of γδ T cells are missing and others as well as αβ T cells exhibit signs of restricted repertoire (8). In our zebrafish mutants, low efficiency of thymopoiesis, biased VDJ rearrangements, aberrant selection of the resulting TCRs, and a low efficiency of rearrangements per se may all contribute to a biased repertoire. It also appears that tcrβ-expressing T cells are more severely affected than tcrδ-expressing cells. An oligoclonal T cell repertoire, defective T cell selection, and impaired CD4 vs CD8 lineage decisions have been found in the thymus of *Ikaros* null mice (40), supporting the view that the functions of Ikaros in T cell development are similar in mouse and fish.

In the mouse, B cell development does not recover in adult *Ikaros* mutants (8). In zebrafish, this is only true for the *igz*-expressing lineage of B cells, while cells expressing the *igm* isotype recover. At present, we do not know whether the recovery of *igz*-expressing B cells is due to the particular nature of the *ikaros* allele or a species-specific difference between zebrafish and mouse. We note, however, that a hypomorphic allele of *Ikaros* has been shown to allow residual B cell differentiation in the mouse (10). If rearrangement at the *tcr* loci is instructive, each cell lineage should rearrange either *z* or *m*. The latter is compatible with our results, suggesting that the development of B cells expressing the *z* and *m* isotypes can be genetically separated with respect to the requirement of normal *ikaros* function.

Ikaros likely functions at different levels of T and B lymphocyte differentiation. First, it regulates the provision of lymphoid progenitors. For larval lymphopoiesis, this function is essential, whereas at later stages of development, *ikaros* function is partially dispensable. Second, *ikaros* appears to participate or be required in lineage decisions among lymphoid subtypes, namely αβ- vs γδ-expressing T cells and *igz*- vs *igm*-expressing B cells. Third, it appears to play a role in the regulation of Ag receptor rearrangements as exemplified by the biased usage of V and J elements in tcrδ rearrangements.

Remarkably, no excessive deaths were observed among mutant fish up to 17 mo of age, suggesting that even a severely restricted Ag receptor repertoire does not impair essential surveillance functions such as specific immune defense and wound repair (data not shown). Interestingly, the oligoclonal repertoire does not lead to overt lymphoma or leukemia and no indolent disease was observed in three 1-year-old mutant fish using complete histological surveys; this may be due to residual activity of the truncated Ikaros transcription factor suppressing uncontrolled proliferation and/or due to the fact that secondary genetic lesions occur at low frequency, if at all, in our mutant fish.

In conclusion, our analysis of the *ikaros* mutant is the first report of the isolation of a lymphocyte mutant in the zebrafish model based on the presence of a specific phenotype. This suggests that the mutagenesis screens conducted in our and other laboratories (13, 14) may reveal further details of the genetic requirements of zebrafish lymphocyte development. Reassuringly, our data indicate that the overall genetic requirements for lymphopoiesis in zebrafish are very similar to those of mammals. This validates the zebrafish system as a tool to identify novel genes involved in early lymphoid development. Lastly, the analytical tools described here will enable a more detailed analysis of other lymphocytic mutants that have been isolated in our and other screens.

Acknowledgments

The large-scale mutant screen was conducted in collaboration with the Tübingen 2000 Screen Consortium, whose members were from the Max-Planck-Institute of Developmental Biology; F. van Bebber, E. Busch-Nentwich, R. Dahn, H. G. Frohnöhöfer, H. Geiger, D. Gilmour, S. Holley, J. Hooge, D. Jüllich, H. Knaut, F. Maderspacher, H. M. Maischein, C. Neumann, T. Nicolson, C. Nüsslein-Volhard, H. H. Roehl, U. Schönberger, C. Seiler, C. Söllner, M. Sonawane, A. Wehner, and C. Weiler; from Exelixis Germany: P. Erker, H. Habeck, U. Hagner, C. Nennen, E. Kaps, A. Kirchner, T. Koblitzek, U. Langeheinrich, C. Loeschke, C. Metzger, R. Nordin, J. Odenthal, M. Pezzuti, K. Schlombs, J. deSatana-Stamm, T. Trowe, G. Vacun, B. Walderich, A. Walker, and C. Weiler. Members of the Freiburg Screening Group were: M. Bialecki, T. Boehm, D. Dickhoff, T. Franz, M. Held, M. Leicht, E. Nold, T. Nolting, C. Riegger, M. Schorpp, and W. Wiest. We thank the Zebrafish Genome Sequencing Project for providing sequence data used here for the characterization of the *tcrb* and *tcred* loci. We are grateful to Donatus Boensch and Oleksandr Kuzmenko for excellent fish care. We thank Annette Haas-Assenbaum, Monika Held, Cristian Soza-Ried, and Tanna Franz for help with the cloning of cDNAs for in situ hybridization, Suzann Beetz for the *pca* probe, and Robert Geissler for help with the mapping panels. We acknowledge the close collaboration with M. Hammerschmidt.

Disclosures

The authors have no financial conflict of interest.

References


