Rapid Mapping of Zebrafish Mutations With SNPs and Oligonucleotide Microarrays


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Rapid Mapping of Zebrafish Mutations With SNPs and Oligonucleotide Microarrays

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Large-scale genetic screens in zebrafish have identified thousands of mutations in hundreds of essential genes. The genetic mapping of these mutations is necessary to link DNA sequences to the gene functions defined by mutant phenotypes. Here, we report two advances that will accelerate the mapping of zebrafish mutations: (1) The construction of a first generation single nucleotide polymorphism (SNP) map of the zebrafish genome comprising 2035 SNPs and 178 small insertions/deletions, and (2) the development of a method for mapping mutations in which hundreds of SNPs can be scored in parallel with an oligonucleotide microarray. We have demonstrated the utility of the microarray technique in crosses with haploid and diploid embryos by mapping two known mutations to their previously identified locations. We have also used this approach to localize four previously unmapped mutations. We expect that mapping with SNPs and oligonucleotide microarrays will accelerate the molecular analysis of zebrafish mutations.

[Supplemental material is available online at www.genome.org. The sequence data described in this paper have been submitted to dbSNP under accession nos. S103507–S105537. The following individuals kindly provided reagents, samples, or unpublished information as indicated in the paper: J. Postlethwait, C.-B. Chien, C. Kimmel, L. Maves, and M. Westerfield.]

Methods

To locate SNPs at defined positions throughout the genome, we sequenced about 1000 PCR fragments, most of which were derived from meiotically mapped ESTs (Kelly et al. 2000; Woods et al. 2000), from the divergent inbred strains C32 and SJD. Because these ESTs were mapped on the basis of genetic polymorphisms (Woods et al. 2000), selection of mapped fragments enriched for SNP-containing sequences. PolyPhred analysis identified 1313 SNPs in 191,005 bp (average frequency 1 SNP/145 bp) of sequence derived from meiotically mapped ESTs (Kelly et al. 2000; Woods et al. 2000). Inspection of 41,640 fragments of mapped ESTs (Woods et al. 2000). Inspection of 41,640 bp of sequence from ESTs that were not known previously to contain polymorphisms revealed 190 additional SNPs (average frequency 1 SNP/219 bp). In surveys of other genes of interest, we identified 384 SNPs between C32 and SJD, raising the total of SNPs characterized in the two strains to 1887 (Fig. 1; Web Supplement A). We also noted 178 insertions/deletions (indels) of 1–6 bp (Web Supplement B).

To measure the rate of polymorphism in other strains (Johnson and Zon 1999), we sequenced a subset of the fragments described above in the AB, TL, Tu, and WIK strains.

Genetic screens in zebrafish (Danio rerio) have isolated several thousand mutations that define the functions of hundreds of essential genes (Driever et al. 1996; Haffter et al. 1996). Identification of the genes disrupted by these mutations can provide molecular entry points into a wide array of biochemical pathways acting in vertebrate development, physiology, and behavior. Although insertional mutagenesis with retroviral vectors has been used in some genetic screens (Golling et al. 2002), the great majority of zebrafish mutations have been induced by the point mutagen ethyl nitrosourea (ENU) (Driever et al. 1996; Haffter et al. 1996). Genes mutated by ENU are identified by the positional cloning and candidate gene approaches, which are critically dependent on knowledge of the mutation’s map position (for review, see Talbot and Hopkins 2000). Current methods involving microsatellite markers have localized some of the mutations identified in the first large-scale screens. Most, however, have not been mapped, and many new mutations are being identified in ongoing genetic screens. Therefore, developing strategies and resources to accelerate the genetic mapping of zebrafish mutations is an important goal.

In human and several model systems, the advent of single nucleotide polymorphism (SNP) maps and high-throughput techniques to rapidly score SNPs has accelerated mapping of mutations (Wang et al. 1998; Winzeler et al. 1998; Cho et al. 1999; Lindblad-Toh et al. 2000; Berger et al. 2001; Hoskins et al. 2001; Wicks et al. 2001; Guo et al. 2002; Swan et al. 2002). Although extensive maps of genes and microsatellites have been constructed for the zebrafish (Postlethwait et al. 1998; Geisler et al. 1999; Shimoda et al. 1999; Barbazuk et al. 2000; Woods et al. 2000; Hukriede et al. 2001), no zebrafish SNP map has heretofore been available. We report the construction of a first generation SNP map of the zebrafish genome. In addition, we describe a method for mapping mutations in which hundreds of SNPs can be scored in parallel by hybridization to an oligonucleotide microarray. By facilitating genetic mapping, this SNP map and corresponding oligonucleotide microarray will accelerate the molecular analysis of zebrafish mutations.

RESULTS AND DISCUSSION

To locate SNPs at defined positions throughout the genome, we sequenced about 1000 PCR fragments, most of which were derived from meiotically mapped ESTs (Kelly et al. 2000; Woods et al. 2000), from the divergent inbred strains C32 and SJD. Because these ESTs were mapped on the basis of genetic polymorphisms (Woods et al. 2000), selection of mapped fragments enriched for SNP-containing sequences. PolyPhred analysis identified 1313 SNPs in 191,005 bp (average frequency 1 SNP/145 bp) of sequence derived from mapped, polymorphic ESTs (Woods et al. 2000). Inspection of 41,640 bp of sequence from ESTs that were not known previously to contain polymorphisms revealed 190 additional SNPs (average frequency 1 SNP/219 bp). In surveys of other genes of interest, we identified 384 SNPs between C32 and SJD, raising the total of SNPs characterized in the two strains to 1887 (Fig. 1; Web Supplement A). We also noted 178 insertions/deletions (indels) of 1–6 bp (Web Supplement B).

To measure the rate of polymorphism in other strains (Johnson and Zon 1999), we sequenced a subset of the fragments described above in the AB, TL, Tu, and WIK strains.
This analysis revealed 148 SNPs that were not identified in the survey of C32 and SJD. In any pair-wise comparison of AB, TL, Tü, and WIK, 30% to 43% of the SNP loci sequenced were polymorphic (Table 1). The polymorphism rate increased in comparisons with C32 and SJD (Table 1), reflecting the bias caused by our selection of fragments known to be polymorphic in these strains.

In total, our sequence comparisons identified 2035 SNPs in 712 genes and ESTs (Web Supplement A). Because the great majority of the SNP-containing fragments had been meiotically mapped in previous studies (Woods et al. 2000), we could determine the map positions for 1930 of the SNPs (Fig. 1; Web Supplement A) and 164 of the indels (Web Supplement B) that we identified. The SNPs occupy 430 unique positions on the female meiotic map (Fig. 1). The average distance between groups of SNPs is 6.98 cM (3000 cM/430 map positions), with the biggest gap spanning 57.9 cM at the top of linkage group 7. Transversions comprise 45.4% of the single base pair substitutions and transitions, 54.6% (Fig. 2). Among transversions, A→T events are over-represented and G→C events under-represented. These figures are quite similar to those reported for Caenorhabditis elegans (Wicks et al. 2001), but differ from other figures published for Drosophila and mammalian SNPs (Hacia et al. 1999; Petrov and Hartl 1999; Lindblad-Toh et al. 2000; Taillon-Miller and Kwok 2000; Berger et al. 2001).

To explore the utility of SNPs in mapping mutations, we developed a strategy using an oligonucleotide microarray to simultaneously score hundreds of SNPs at defined genomic locations. In this method, SNPs are scored by discrimination between perfect matches and single base pair mismatches in hybridization assays with oligonucleotide probes. The microarray comprised oligonucleotide probes (10–26 bp) predicted to have the same Tm (50°C) for all four possible alleles for each of 599 SNPs derived from the C32-SJD sequence survey (Fig. 3A; Web Supplement A). The 599 SNPs define 324 genes/ESTs at 234 unique map positions on the female meiotic map (Fig. 1; Web Supplement A), such that the average distance between groups of SNPs represented on the array is 12.8 cM (3000 cM/234). With respect to the sex-averaged map (Shimoda et al. 1999), which is more relevant for genetic mapping in standard diploid crosses, the average distance between groups of SNPs on the array is 9.8 cM (2300 cM/234).

We used this array to map zebrafish mutations with a variation of the bulked segregant analysis approach that is commonly used in zebrafish mapping projects. In the traditional approach, gel-based markers are scored in pools of wild-type and mutant genomic DNA, and differential amplification of alleles in the two pools identifies linked markers (Postlethwait et al. 1994; Talbot and Schier 1999). In the array approach, fragments encompassing each SNP are amplified by PCR from pools of wild-type and mutant genomic DNA (Fig. 3B). The two pools of DNA fragments are differentially labeled with Cy3 and Cy5, made single-stranded, and simultaneously hybridized to the array (see Methods). The results of the hy-

<p>| Table 1. Percentage of Analyzed SNPs That are Polymorphic Between Common Zebrafish Strains |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Strain</th>
<th>C32</th>
<th>SJD</th>
<th>WIK</th>
<th>Tü</th>
<th>TL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>41.8% (207/495)</td>
<td>64.2% (325/506)</td>
<td>34.2% (149/436)</td>
<td>30.2% (121/401)</td>
<td>36.5% (155/425)</td>
<td></td>
</tr>
<tr>
<td>TL</td>
<td>44.3% (224/506)</td>
<td>59.0% (301/510)</td>
<td>43.0% (187/433)</td>
<td>29.9% (119/398)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tü</td>
<td>46.5% (214/460)</td>
<td>60.0% (290/483)</td>
<td>36.2% (149/412)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WIK</td>
<td>53.9% (269/499)</td>
<td>53.8% (275/511)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SNP-containing fragments from single adult fish of the indicated strains were amplified by PCR and sequenced. Note that the analyzed fragments were prescreened for polymorphisms between C32 and SJD.
bридизацию аре then quantified with a microarray scanner, and base calls for each SNP are generated from the relative fluorescence intensity of the four probes for that SNP (Fig. 3C). When haploids are used for mapping, SNP loci linked to the mutation locus exhibit differential labeling of alleles (Fig. 3B; C in wild type, T in mutant), whereas Cy3 and Cy5-labeled DNA hybridize equally well to both alleles of unlinked SNPs (Fig. 3B; T and A alleles). Because not all SNPs are informative in every cross, monomorphic loci are also detected and are visible as a single spot with both labels at one base position on the array.

As a test of the array mapping approach, we mapped floating head (flh), a mutation localized previously to linkage group (LG) 13 (Talbot et al. 1995). We analyzed a haploid mapping cross between the outbred flh line and the DAR strain (Johnson and Zon 1999), which shows a high degree of polymorphism compared with commonly used lab strains (Fig. 3C). Of the 599 SNPs on the array, one SNP (ZSNP1100) near the middle of LG13 displayed differential labeling indicative of linkage. Restriction fragment length polymorphism (RFLP) analysis on the individual embryos contributing to the pooled DNA samples demonstrated that this SNP was located 19 cM from flh. In addition, the array generated unambiguous base calls for 324 other SNPs, which exhibited labeling characteristic of polymorphic unlinked (80) or monomorphic (244) loci. No false positives or false negatives were evident.

The same general principles apply to the use of these microarrays with diploid embryos, although the analysis is slightly different due to the presence of embryos heterozygous for the mutation in the wild-type pool (Fig. 4A). Because of these heterozygotes, SNP alleles linked in cis to the mutation are found in both pools, whereas SNP alleles tightly linked in cis to the wild-type form of the gene are present only in the wild-type pool. Thus, only probes for alleles linked in cis to the wild-type allele of the gene of interest display differential labeling.

Figure 4 depicts the mapping results for st11, a previously unmapped mutation disrupting notochord differentiation that we identified in a screen for mutants with embryonic lethal phenotypes (I.G. Woods and W.S. Talbot, unpubl.). To map st11, which was isolated in a TL background, we analyzed diploid F2 embryos from a cross constructed with the WIK mapping strain (Johnson and Zon 1999; Neshporuk et al. 1999). In an analysis of 599 SNPs, six SNPs located on LG2 showed differential labeling between wild-type and mutant pools (Fig. 4B; data not shown). Five of these SNP clusters in the middle of the linkage group (Fig. 4C). Eleven other SNPs also exhibited differential labeling indicative of possible linkage, but the map positions of these were scattered throughout the genome and three of the eleven were located in the same gene or EST as a SNP with labeling characteristic of unlinked SNPs. All other SNPs that generated unambiguous base calls (450) exhibited labeling characteristic of polymorphic unlinked (56) or monomorphic (394) loci. Hence, analysis of the array data suggested that the st11 gene is located on LG2, and that the putatively linked markers on other linkage groups were false positives. This interpretation was verified by scoring RFLPs caused by three of the LG2 SNPs in individual st11 mutants and wild-type siblings; these markers were located 2–15 cM from the st11 mutation (Fig. 4C). In addition, analysis of SSLP markers on LG2 indicated that st11 was located within the cluster of five linked SNPs (Fig. 4C). The sixth SNP locus on LG2 (ZSNP196) lies –70 cM from the mutation and is separated from the mutation by a putatively linked SNP, suggesting that it is most likely an additional false positive. Parsons et al. (2002) reported recently that sleepy, a mutation disrupting notochord differentiation, maps to the same region of LG2. Our map assignment and the phenotypic similarity suggest that st11 is a new allele of sleepy.

We have used the microarray strategy to map four other mutations to the correct location in diploid crosses, including iguana, which was mapped previously to LG6 (H. Stickney and W. Talbot, unpubl.), and three other mutations for which no prior map information was available. In these experiments, genotypes were assigned for 60.0% (1361/2269) of the SNPs analyzed. The polymorphism frequency ranged from 17.4%–28.7%, corresponding to an average of 77 polymorphic markers per mapping experiment (range 62–88). One to four linked markers were detected in each experiment, and the false positive rate was 2.5% (34/1361).

We have constructed the first SNP map of the zebrafish genome and developed a new strategy to localize zebrafish mutations by scoring SNPs from pooled genomic DNA samples with oligonucleotide microarrays. Because SNPs can be scored with high-throughput methods (Kwok 2001), SNP maps have accelerated genetic mapping in a variety of model organisms (Winzeler et al. 1998; Cho et al. 1999; Lindblad-Toh et al. 2000; Berger et al. 2001; Hoskins et al. 2001; Wicks et al. 2001; Swan et al. 2002). For example, a mouse SNP map with 1942 mapped markers and a multiplex genotyping assay allow mouse mutations to be mapped in a genome scan with only six genotyping reactions per animal (Lindblad-Toh et al. 2000). Similarly, our zebrafish SNP map comprising 2102 markers enables rapid mapping of zebrafish mutations. The microarray approach that we have developed simultaneously scores hundreds of SNPs in two samples, wild-type and mutant DNA pools, on a single microarray. Differential labeling on alleles of linked markers suggests possible map locations, which can then be tested by scoring markers in the region (e.g., mapped SNPs or SSLPs) on individuals. Analysis of pooled samples with the microarray can detect linkage of markers 10–20 cM from the mutation (Figs. 3 and 4; data not shown). This indicates that, under ideal conditions, analysis of only 60–120 polymorphic SNPs spaced at intervals of 20–40 cM over the 2300 cM sex-averaged map (Shimoda et al. 1999) would be sufficient to identify a marker linked to the average mutation. In practice, it is necessary to score more markers, because the SNPs on the current version of the microarray are not evenly spaced and only a fraction of the SNPs are infor-
mative in the outbred backgrounds commonly used for map-
ing crosses (see Table 1). We expect that future versions of
the array will feature improvements in coverage and poly-
morphism rate of the represented mark-
ers. Nonetheless, the current array
represents 599 SNPs, and our analy-
sis of a variety of genetic back-
grounds indicates that the array
will be useful in most crosses with
commonly used mapping strains.
In combination with the advancing
genomic sequence and the ability
to rapidly test candidate genes,
mapping with SNPs and oligo-
nucleotide arrays will accelerate the
molecular analysis of zebrafish mu-
tations.

METHODS

Fish Strains

The derivation of fish strains C32,
SJD, AB, TL, Tü, and WIK has been
described (Haffter et al. 1996;
Johnson and Zon 1999). Genomic
dNA samples from C32 and SJD
fish were kindly provided by J.
Postlethwait (Univ. of Oregon). Ge-
nomic DNA from the other strains
was prepared from fish stocks main-
tained in our facility at Stanford.
C32 and SJD are partially inbred
lines, and previous work showed
that these strains are not com-
pletely homozygous for all genetic
markers (Nechiporuk et al. 1999).
Accordingly, we found that a C32
adult was heterozygous for 92 of
1887 SNPs analyzed, and a SJD
adult was heterozygous for 27 of these
1887 SNPs (Web Supplement A).

SNP Sequencing and Detection

To identify SNPs, fragments from
C32, SJD, AB, TL, Tü, or WIK ge-
nomic DNA were amplified by PCR
and directly sequenced on both
strandswith an ABI377 or ABI3700.
Most of the primers were designed
from 3’/H11032 ESTs for previous mapping
experiments (Woods et al. 2000),
but some were designed specifically
to analyze SNPs of interest. Se-
quence traces were analyzed with
Phred (Ewing et al. 1998) and as-
sembled with Phrap (www.phrap.
org). Polyphred (Nickerson et al.
1997) was used to identify puta-
tive SNPs. Polyphred ranked 1–3
SNPs were inspected with Consed
(Gordon et al. 1998) and called
directly from the traces. Some of
the traces were also examined
using DNAstar software to identify
SNPs.

Oligonucleotide Microarrays

The oligonucleotide microarrays
were constructed by Protogene, Inc., using ink-jet in situ syn-
thesis and differential surface tension technology (Butler et al.
2001).
Hybridization Assays

We prepared genomic DNA from 16–50 mutant embryos and a similar number of wild-type siblings from a mapping cross as described (Talbot and Schier 1999). PCR primers were designed with Primer 3 (Rozen and Skaletsky 1998) to have a length of 21–27 nucleotides, a Tm of 57–63°C, and a product of 100–175 bp. One primer from each pair was phosphorylated at the 5’ end with T4 polynucleotide kinase. Multiplex (4X) amplification reactions were performed in a 25 µL volume containing ~13 ng genomic DNA, 0.3 µM primers, 0.6 U Taq DNA polymerase, 100 µM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.0001% gelatin, and 0.01 mg/mL BSA. Thermocycling was performed under standard conditions consisting of an initial denaturation at 94°C for 2 min, followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 30 sec, and then a final incubation at 72°C for 5 min.

Following amplification, the PCR products were purified with QIA Gen miniprep columns. Cyanine 3-dUTP or Cyanine 5-dUTP fluorescent labels from NEN Life Science Products, Inc were attached to the 3’ end of the purified PCR product with Takara terminal deoxynucleotidyl transferase (0.4 mM Cyanine label per 40 pmoles DNA, 2.5 mM CoCl2, 0.2M potassium cacodylate, 25 mM Tris-HCl, and 0.25 mg/mL BSA; overnight reaction at 37°C). One strand was digested with Lambda exonuclease (5 units Lambda exonuclease per 2 µg DNA, 67 mM Glycine-KOH, 2.5 mM MgCl2, 0.25 µg/mL BSA; 1-h incubation at 37°C). The single-stranded product was then reduced to less than 3 µL by use of a YM-30 Microcon filter device and brought to a final volume of 35 µL by addition of hybridization solution.

The array was pre-soaked in hybridization solution containing 50 mM 2-[N-morpholino]ethanesulfonic acid (MES), 250 mM NaCl, and 0.1% Tween-20 at room temperature for 20 min, and allowed to air dry. The labeled sample was denatured at 95°C for 10 min, immediately cooled in an ethanol-dry ice bath, and allowed to thaw on ice. The 35-µL sample was then applied to the array and incubated at 42°C for 1 h. After hybridization, the array was washed once at 42°C for 5 min with a solution of 2× SSC and 0.1% Tween-20, followed by a 5-min wash at 42°C with 1× SSC and 0.1% Tween-20. The array was scanned at 532 and 635 nm with a GenePix 4000a microarray scanner.

GenePix software was used to identify SNPs and microarrays in Zebrafish.
generate a digitized intensity table for each of the features on the array. An Excel macro in combination with visual inspection of the intensity data was then used to score the SNPs. The macro requires three calls by first establishing background levels for each SNP by averaging the intensity levels on the two alleles with the least hybridization. This background level is then subtracted from the hybridization intensity for the two remaining alleles. If the adjusted intensity of the allele with the most hybridization is more than twice that of the adjusted intensity of the remaining allele, the macro calls that allele; otherwise the macro calls both bases.

**SSLP Markers**

SSLP analysis was performed as described (Tabbot and Schier 1999) to confirm locations derived from microarray data for previously unmapped mutations.

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