Production of Androgenetic Zebrafish (*Danio rerio*)

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ABSTRACT

To help investigate the evolutionary origin of the imprinting (parent-of-origin mono-allelic expression) of paternal genes observed in mammals, we constructed haploid and diploid androgenetic zebrafish (*Danio rerio*). Haploid androgenotes were produced by fertilizing eggs that had been X-ray irradiated to eliminate the maternal genome. Subsequent inhibition of the first mitotic division of haploid androgenotes by heat shock produced diploid androgenotes. The lack of inheritance of maternal-specific DNA markers (RAPD and SSR) by putative diploid and haploid androgenotes confirmed the androgenetic origin of their genomes. Marker analysis was performed on 18 putative androgenotes (five diploids and 13 haploids) from six families. None of 157 maternal-specific RAPD markers analyzed, some of which were apparently homozygous, were passed on to any of these putative androgenotes. A mean of 7.7 maternal-specific markers were assessed per family. The survival of androgenetic zebrafish suggests that if paternal imprinting occurs in zebrafish, it does not result in essential genes being inactivated when their expression is required for development. Production of haploid androgenotes can be used to determine the meiotic recombination rate in male zebrafish. Androgenesis may also provide useful information about the mechanism of sex determination in zebrafish.

*Zebrafish* (*Danio rerio*, formerly known as *Brachydanio rerio*; MEYER et al. 1993) are an important model for studying vertebrate development and are amenable to genetic analysis (STREISINGER et al. 1981; KIMMEL 1989; BARINAGA 1990, 1994; NÜSSLIN-VOLHARD 1994; CONCORDET and INGHAM 1994; DRIEVER et al. 1994; KAHN 1994). Their use as a model organism for genetic analysis is facilitated by a linkage map of DNA markers (POSTLETHWAIT et al. 1994) and large scale screens for mutations are underway (DRIEVER et al. 1994; KAHN 1994; MULLINS et al. 1994).

To investigate imprinting in vertebrates and to help develop zebrafish as a genetic system, we constructed haploid and diploid androgenotes. Construction of individuals with uniparental inheritance can facilitate genetic analysis. Haploid and diploid gynogenotes have been produced by fertilizing zebrafish eggs with sperm irradiated to eliminate the paternal genome (STREISINGER et al. 1981; HÖRSTGEN-SCHWARK 1993). Haploid gynogenotes were used to produce a zebrafish linkage map based on rates of meiotic crossing over in oocytes (POSTLETHWAIT et al. 1994). Haploid gynogenotes complete embryogenesis and arrest as larvae. Thus, haploid embryos can be used for F1 mutant screening (KIMMEL 1989): mutations in the stem cells of the maternal germ line are introduced by fertilization with mutagenized sperm or by mutagenesis of early embryos. Such screens require maintenance of considerably fewer progeny to recover an interesting recessive mutation from the maternal stock than conventional diploid screens, which require production of an F3 generation (KAHN 1994; MULLINS et al. 1994). Diploid gynogenotes can be produced by inhibiting extrusion of the second polar body or by inhibiting the first mitotic division of the gynogenote (STREISINGER et al. 1981; HÖRSTGEN-SCHWARK 1993). In the latter instance, the progeny are homozygous and clonal lines of gynogenetic zebrafish have been produced (STREISINGER et al. 1981; KIMMEL 1989).

Androgenetic haploid progeny would result from fertilization of eggs that have been treated to eliminate the maternal genome. A method for production of androgenetic zebrafish has not been reported, but would facilitate determination of rates of meiotic recombination in males, mapping of male specific DNA markers and linkage groups (if any), and analyses of sex determination and genomic imprinting. Use of androgenetic haploids may have some usefulness for F1 mutant screens if interesting mutations can be reliably recovered from cryopreserved milt long after the screen was performed.

Like haploid gynogenotes, haploid androgenotes are expected to arrest after embryogenesis, but inhibition of the first mitotic division should produce homozygous diploid androgenotes. A major technical impediment to producing androgenetic zebrafish has been the short period of time after egg collection during which successful fertilization can be accomplished. This restricts the opportunity for manipulations, such as irradiating to destroy the maternal genome. Other technical impediments include the possibility that irradiation of eggs might damage the egg cytoplasm, maternal RNA,
or mitochondrial DNA. Genomic imprinting of essential genes that are irreversibly suppressed at a required developmental stage and derived from the paternal genome as in mammals (McGrath and Solter 1984; Surani et al. 1984; Surani 1986; Barra and Renard 1988; Sapienza 1990; Renard et al. 1991; Gold and Pederson 1994; Ghai let et al. 1995) would make the survival of androgenotes impossible. Recessional lethal mutations could limit the successful production of androgenotes, but this is not expected to be an insurmountable problem since gynogenetic homoyzogous zebrafish have been produced (Streisinger et al. 1981; Horstgen-Schwark 1993), and inbred lines are available. We present genetic evidence that haploid and diploid androgenetic zebrafish can be constructed. To confirm androgenesis, lack of inheritance of maternal markers is a crucial part of the analysis. Markers relying on gene expression (e.g., phenotypic traits, isozymes, and allozymes) can be affected by many factors including imprinting, tissue specific expression, and developmental specific expression. Failure to detect a maternal marker that results from gene expression in a putative androgenote can be attributed to lack of maternal DNA in the putative androgenote or to lack of expression. Thus, we directly assayed the DNA of putative androgenotes for maternal specific markers using PCR methods.

METHODS AND MATERIALS

Production of androgenetic fish: Androgenetic haploids are produced by irradiating eggs to destroy the maternal genome followed by fertilization. By inhibition of the first mitotic division, diploid androgenotes can be produced.

A Torrex 150D X-ray inspection system (Faxitron X-Ray Corp., Buffalo Grove, IL) was used to irradiate eggs. X-ray dosimetry was performed with a MDH model 10x5-180 ion chamber (paddle chamber). This was calibrated with a known 137Cs source (NBS source No. 47455). The appropriate dose to eliminate the maternal DNA was determined based on the Hertwig effect (Hertwig, 1911). Eggs were collected from fish anesthetised in 17 ppm (w/v) Tricaine (3-aminobenzoic acid ethyl ester, Sigma A5040; pH adjusted to ~7 with sodium bicarbonate) by gently squeezing the abdomen. Eggs were collected into a silanized capillary tube and placed into ~100 µl of coho salmon (Oncorhynchus kisutch) ovarian fluid (the fluid surrounding mature eggs) in a petri dish. The milt was collected just prior to being used for fertilization and was held in sperm extender: 80 mM KCl, 45 mM NaCl, 0.4 mM CaCl2, 0.2 mM MgCl2, 45 mM sodium acetate, and 10 mM HEPEs, pH 7.7 (Gibbs et al. 1994).

In the first experiment, eggs were collected from one female, and divided into eight groups. Each group of ~100 (88–112) eggs was held in coho salmon ovarian fluid and was exposed to a different total accumulated irradiation dose. All eggs were simultaneously inseminated, and survival was scored at 1 day postfertilization (p.f.). In the second trial, five groups of eggs (69–102 eggs) from a single female were irradiated with different total accumulated doses of X-rays. Living embryos were scored at 1 day p.f. and at 4 days p.f. according to appearance. Based on the results of these two dose response trials, a dose of 10,000 R was used in the following experiments to produce androgenotes.

The first mitotic division was inhibited by heat shock treatment. After fertilization, eggs were held at 28.5 ± 0.5°C for 15 min, then heat shocked for 2 min at 41.4 ± 0.03°C, and returned to 28.5°C (modified from Streisinger et al. 1981). Temperatures were measured with a calibrated thermometer (Fisher Scientific, Cat. No. 15041A) having an uncertainty certified not to exceed 0.05°C.

Families analyzed: Fish from two laboratory lines of fish were used: *AB line (star AB line), which has been screened for recessive lethals (C. Walker, personal communication) and the SFU line, which originated from zebrafish bought from pet stores on Vancouver Island, B.C., Canada.

Production of haploid or diploid androgenotes was attempted in 14 families (two families used for Hertwig experiments plus families A–H, Tables 4 and 5, plus seven other families). In six of these families (Table 5), inheritance of parental DNA markers was assessed in a sample of the putative androgenetic progeny, with family A being subjected to the most thorough genetic analysis.

Family A: Eggs were collected from one female of the SFU line, and the milt was from one male of the *AB line. The eggs were held in coho salmon ovarian fluid at room temperature for 50 min, the time required for irradiation of eggs. Of the 280 eggs collected, 76 eggs were not X-ray irradiated (NI) and 204 eggs were irradiated (I) with 10,000 R of X-rays. All eggs were then simultaneously inseminated. Of the irradiated eggs, 49 were not heat shocked (I/NHS; treatment to produce putative haploid androgenotes: PHA) and 155 were heat shocked (I/HS; treatment to produce putative diploid androgenotes: PDA). The NI/NHS (not irradiated and not heat shocked) treatment was used to produce normal biparental diploid progeny: BDP.

DNA extraction: The following tissue samples were collected to prepare DNA extracts: both parents (caudal fin clips); 12 biparental diploid progeny (whole fish collected at 5 days postfertilization (p.f.)); two putative haploid androgenotes (whole fish collected at 5 days p.f.); one putative diploid androgenote (whole fish at 9 days p.f.); and a second putative diploid androgenote (caudal fin clip at 1.5 months p.f.). Whole fish were collected before feeding to decrease DNA contamination. All tissue samples were thoroughly rinsed with pure water before DNA extraction. DNA was prepared by phenol/chloroform extraction as described by Sambrook (1989). The protocol was slightly modified by extracting twice with phenol, twice with 1:1 phenol/chloroform and once with chloroform; DNA precipitation was done by adding one-tenth volume 2.5 M sodium acetate (pH 5.5) and 2 volumes cold absolute ethanol. Quantification of DNA was done by spectrophotometry using Pico-Green (Molecular Probes, Inc., Eugene, OR) on a SLM 4800 C Subnanosecond Spectrophotometer (SLM Aminco, SLM Instruments Inc., Urbana, IL). DNA quantification standards were prepared from calf thymus DNA (Sigma, D 1501).

Fluorescent DNA Primers: RAPD (random amplified polymorphic DNA) (Williams et al. 1993) oligonucleotide primers were synthesized and fluorescently labeled with 6-FAM on an ABI 392 DNA/RNA synthesizer (Applied Biosystems Inc.). FAM amide (6-FAM) is a carboxyfluorescein derivative and was attached to the 5’ end of the primer by amino linker chemistry according to ABI user bulletin number 67. Quantification of fluorescent primers was based on absorbance at 260 nm using a Beckman DU640 Spectrophotometer. Primers were diluted to their working concentration of 5.0 µM in pure water and stored frozen. RAPD primer 208BCF is AGGCGGACCC; RAPD primer 210BCF is GCAAGGAGAG, and RAPD primer 289BCF is CCAGTTGCC. These sequences correspond to RAPD primers numbers 208, 210 and 269, respectively, that were produced by Nucleic Acid-Protein Service.
PCR conditions: For RAPD amplifications, 4 ng of each template DNA was added to an aliquot of PCR cocktail containing: 1X Idaho 5 mM Mg buffer (Idaho Technology Inc., Idaho Falls, ID), 100 μM of each dNTP (Pharmacia) and 0.06 U/μl of Taq DNA polymerase (Promega in storage buffer B) and 1.6 μM of one of the three fluorescently labeled 10-mer primers. Thermocycling was performed in heat-sealed glass capillary tubes containing a total volume of 10 μl in an Idaho 1605 Air-Thermo-Cycler. Two cycles of 91° for 60 sec, 42° for 7 sec, and 72° for 70 sec was followed by 38 cycles of 91° for 1 sec, 42° for 7 sec, and 72° for 70 sec, which was followed by a 5-min hold at 72°. SSR (simple sequence repeat) conditions were identical except that annealing was at 69° for 10 sec.

Analysis of RAPD-PCR products: Aliquots of each PCR reaction were separated by electrophoresis on agarose and polyacrylamide gels. One aliquot of each PCR reaction was loaded onto 1.8% agarose gels in 0.5× TBE (0.045 M Tris base, 0.045 M boric acid, 0.001 M EDTA) containing 0.5 μg/ml of ethidium bromide. Electrophoresis was performed at 3 V/cm. Ethidium bromide staining was visualized with a 300-nm transilluminator, and the images were captured on a UVP Gel Documentation System (UVP, San Gabriel, CA). These images were analyzed using the NCSS GelReader program, Macintosh version 2.0.5 (National Center for Supercomputing Applications, University of Illinois at Urbana-Champaign).

The second aliquot of each RAPD-PCR reaction was loaded onto a 4% native polyacrylamide gel on an ABI 373 DNA Sequencer. ABI’s fluorescent ROX-GS2500 inlane size standard was also loaded onto all lanes. Data were collected with the ABI GeneScan Collection Software (version 1.1) and analyzed with the ABI GeneScan Analysis Software (version 1.2.2.1). RAPD markers are dominant markers and were scored as either present or absent. By scoring for presence or absence of each marker in 12 normal biparental diploid progeny, it was determined if the marker was heterozygous or apparently (see below) homozygous in the parent. To check that the markers were segregating in a normal Mendelian fashion, for each of the heterozygous markers the number of BDP containing that marker were scored. The observed present:absent ratio in the F1 was then checked against the theoretical ratio of 1:1 using chi-square analysis, employing the Yates correction for continuity. To check that markers were assorting independently, each heterozygous marker was then tested for cis or trans linkage with each other marker. Markers were named according to the convention adopted for the zebrafish RAPD linkage map (POSTLETHWAIT et al. 1994): markers are prefixed with the RAPD primer used and suffixed with the size designation obtained from the Genescan software. For example, 210BF6453 is a marker amplified with the sequence of primer 210 from the UBC primer series, the F designates a FAM labeled primer, and 453 designates the size of the amplified fragment.

MHC and SSR pedigree analysis: To further investigate inheritance to putative androgenetic progeny, other PCR-based methods were used to screen for polymorphisms between parental DNAs. The MHC (Major Histocompatibility Complex class II genes) primers Tu360 and Tu385 (ONO et al. 1992) were used as they often show polymorphisms between the AB line of zebrafish and other zebrafish strains (POSTLETHWAIT et al. 1994). We also used a set of 16 forward and reverse simple sequence repeat (SSR) primers (GOFF et al. 1994). The fragments amplified by the MHC primer Tu360 and Tu385, and the fragments amplified by the SSR primers have been placed on the zebrafish linkage map (POSTLETHWAIT et al. 1994).

RESULTS

Irradiation dosage: In the first Hertwig dose response survival trial (Figure 1), a shoulder was observed at 10,000 R. Survival in the first trial included embryos that developed as masses of cells without any discernible body axis at 24 hr p.f. In the second trial, only those surviving embryos that developed a body axis are represented in Figure 1. For 5000 R dosage group at 24 hr, 5% had developed a body axis and 52% had developed as a mass of cells with no body axis. These were both scored as alive in experiment 1. When viewed at 4 days p.f., in the unirradiated group, all embryos alive at 24 hr were still alive and all appeared to be normal diploids (see below and Figure 2), the group irradiated with 500 R had four surviving embryos that appeared to be normal diploids, and the rest of the group had moderate to severe abnormalities, none of which displayed the haploid syndrome (see below). All embryos from the 5000 R group were dead and had arrested as grossly abnormal embryos. In the 10,000 R group, all embryos displayed the haploid syndrome (see below), 13 appeared to be normal haploids and 12 appeared to be anatomically abnormal haploids. In the 15,000 R group, 1 normal haploid and 13 abnormal haploids were seen. No embryos with a diploid appearance were seen in the 5000, 10,000, nor 15,000 R groups. Based on these results, 10,000 R was used in later experiments to produce androgenotes.

Production of androgenotes: No embryos with a diploid phenotype were observed among 49 eggs for the 1/NHS (irradiated and not heat shocked) group and two were observed among the 155 eggs for the 1/HS (irradiated and heat shocked) group (Table 1). A syn-
drome similar to the haploid syndrome of gynogenetic haploids (Streisinger et al. 1981; Horstgen-Schwark 1993) was seen at 24 hr as a shortened body phenotype (Figure 2), which was obvious at 48 hr in I/NHS embryos. Melanocytes are characteristically smaller in haploid embryos. This became noticeable at 48 hr (Figure 2) and was pronounced by 96 hr (not shown). The development of putative androgenetic diploid embryos was initially slightly retarded (Figure 2). However, by the end of the first month, the PDA fish in this experiment, and several in other experiments, were approximately the same size as the diploid control fish. In this experiment, the percentage of haploid and diploid androgenotes produced relative to our control group was 14% and 2%, respectively.

**Evaluation of the pedigree analysis technique:** Analysis of DNA polymorphisms was used to determine the inheritance of maternal and paternal DNA to putative androgenetic offspring. Using RAPD primer 208BCF, three maternal-specific markers, but no paternal-specific markers, were detected by agarose gel electrophoresis (Figure 3). None of the maternal markers was inherited by any of the four putative androgenetic progeny. The ABI 373 Automated DNA Sequencer allows for the separation of PCR products with greater resolution and sensitivity than agarose electrophoresis, and the use of infuse fluorescent size markers allows for more precise sizing of fragments, facilitating identification of markers. Thus, most of our genetic analyses were based on fluorescent RAPD products separated on the ABI sequencer.

Figure 4 shows output from the Genescan program. A comparison of two separate RAPD-PCR reactions replicated for each of two DNA templates (parents of putative androgenotes) using the same fluorescent primer are shown. Although some peak heights vary slightly, all major peaks can be seen in both PCR reactions that contained an aliquot of the same DNA template, demonstrating that fluorescent RAPD-PCR markers are amplified reproducibly and that they can be reproducibly detected. While the resolution is much better than on agarose gels, some peaks overlap. The zoom feature in the GeneScan software allows resolution of more peaks than can be seen in Figure 4. For our analysis, we used only those markers that were clearly distinguishable. In all the ABI GeneScan electropherograms we have viewed to date, we have never detected a RAPD-PCR product in a progeny that was not detected in one of the parents, which is consistent with our RAPD markers acting as Mendelian markers. A clearly polymorphic peak specific to the father is seen in the top two panels at 799 bp in Figure 4. A small amount of amplification product was observed when no template was included in the PCR reaction (Figure 5). Amplification products appearing in the absence of template DNA, which disappeared when template DNA was included in the PCR reaction, have been previously noted for RAPD reactions (e.g., Williams et al. 1990). PCR markers used in our analyses are clearly distinct in mobility from those amplified in the absence of DNA template.

Examples of a maternal marker and a paternal marker and their inheritance to a normal diploid progeny and a putative diploid androgenetic progeny are shown in Figure 5. The maternal-specific marker 210bcf.453 and the paternal-specific marker 210bcf.799 shown in Figure 5 were found in all 12 biparental diploid progeny tested (only one of which is shown in

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial group size (No. of eggs)</th>
<th>Surviving at 24 hr</th>
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<tbody>
<tr>
<td></td>
<td>Haploids (No. of embryos)</td>
<td>Diploids (No. of embryos)</td>
</tr>
<tr>
<td>NI/NHS</td>
<td>76</td>
<td>0</td>
</tr>
<tr>
<td>I/NHS</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>I/HS</td>
<td>155</td>
<td>0</td>
</tr>
</tbody>
</table>

*I, irradiated; NI, not irradiated; HS, heat shocked; NHS, not heat shocked. Data for family A.*
Inheritance of three maternal polymorphic markers for RAPD primer 208BCF. The inheritance of three maternal RAPD markers is shown for 12 biparental diploid progeny and four putative androgenetic progeny. The PCR products were separated by electrophoresis on a 1.8% agarose gel and stained with ethidium bromide. PHA, putative haploid androgenote (1 and 2); PDA, putative diploid androgenote (1 and 2); C, control (no template); S, sizing standard ( Gibco BRL, 100-bp ladder); ▶, presence of band; ▾, absence of band. Three bands (RAPD markers) in the maternal (♂, ♀) lane are marked with ▶. These bands are absent from the paternal (♂, ♀) lane and were designated as maternal. One of the maternal markers is seen in only some of the biparental diploid progeny and is thus considered heterozygous in the female parent. Two of these maternal bands are seen in all 12 biparental diploid progeny and thus are presumed homozygous in the female parent. None of these three maternal markers was detected in any of the four putative androgenotes tested.

Inheritance of RAPD-PCR markers by putative androgenotes: Using three fluorescent primers, 16 maternal (11 heterozygous and five apparently homozygous) and seven paternal (four heterozygous and three apparently homozygous) markers were identified (Table 2). Markers were considered heterozygous if only some of the progeny received the marker and apparently homozygous if all 12 test BDP received the marker (Table 2, footnote a).

To test whether these RAPD markers were segregating in a Mendelian fashion, the 11 heterozygous maternal

![Diagram](image-url)
markers were scored in the 12 BDP, and the four heterozygous paternal markers were scored in the 16 progeny (12 BDP + two PHA + two PDA). For each marker, the presence/absent count in the F₁ progeny was tested using chi-square analysis for goodness of fit (ZAR 1974) to the theoretical ratio of 1:1. The null hypothesis of no difference to a 1:1 ratio was not rejected, with α = 0.05, for 14 of the 15 heterozygous markers. Thus, 14 of the 15 heterozygous markers appear to behave as Mendelian factors in our analysis. Marker 269BCF:793 was clearly present in both replicates for the maternal template and was clearly present in the one BDP progeny it was observed in. It was considered a statistical outlier and was not excluded from the data. Its exclusion would have very little effect on our androgenetic analysis.

It is important for our analysis to show that each marker represents a different locus, rather than some of them being length variants of the same locus. The 11 maternal heterozygous markers were tested for independent assortment against all other maternal markers. A similar analysis was performed for the four heterozygous paternal markers. For this analysis, it was assumed there was a recessive (unamplified) allele for each of the dominant RAPD markers. As the markers are dominant and parent specific, the cross can be viewed as a test cross. By arbitrarily assigning the two markers being compared as A and B, we use the notation for the cross as AaBb X aabb. If unlinked, the four categories in the cross will have a ratio approximating 1:1:1:1. If the two dominant markers are closely linked on the same chromosome (cis-linked), the AaBb and aabb categories would strongly dominate. If the dominant form of A is

**TABLE 2**

Number of parentally polymorphic RAPD markers in family A

<table>
<thead>
<tr>
<th>Primer</th>
<th>Maternal markers</th>
<th>Paternal markers</th>
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<tbody>
<tr>
<td></td>
<td>Homozygous*</td>
<td>Heterozygous</td>
</tr>
<tr>
<td></td>
<td>Homozygous</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>208BCF</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>210BCF</td>
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<td>5</td>
</tr>
<tr>
<td>269BCF</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

*Markers were designated homozygous if they occurred in all 12 biparental diploid progeny tested. The probability (P) of making an error by calling a marker homozygous, based on it being found in 12 BDP, was calculated: \( P = \frac{1}{n} \) with \( n = \) number of progeny tested. \( P = \left(\frac{1}{12}\right)^{12} = 0.00024414 \). Thus, the chance that a marker designated as homozygous is in fact heterozygous, is 0.00024414 and the chance that it is homozygous is \( (1 - 0.00024414 = 0.99975586) \).
TABLE 3
Inheritance of heterozygous paternal markers by four putative androgenetic progeny tested in family A

<table>
<thead>
<tr>
<th>Marker</th>
<th>PHA</th>
<th>PDA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>210bf.237</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>210bf.313</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>269bf.615</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>269bf.637</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

210BCF, the fluorescent RAPD primer used and the digits after the decimal indicate the size of the amplified fragment; PHA, putative haploid androgenotes (Nos. 1 and 2); PDA, putative diploid androgenotes (Nos. 1 and 2): +, marker present; −, marker absent.

on the same chromosome as the recessive form of B (trans-linked), the Aabb and aaBb categories would strongly predominate. In the 55 comparisons done between maternal heterozygous markers, only one pair of markers appeared cis-linked and this was not complete. The degree of linkage was not calculated due to the small sample size. None of the paternal marker comparisons indicated linkage. In summary, our heterozygous RAPD markers appear to be segregating as Mendelian markers and (with perhaps one exception) appear to be assorting independently.

To verify the androgenetic nature of PHA and PDA progeny of family A, the inheritance of maternal and paternal markers by these progeny was analyzed. All three of the homozygous paternal markers were inherited by all four of the putative androgenetic progeny tested, whereas none of the 16 maternal markers, five of which are probably homozygous, were detected in any of the four putative androgenetic progeny. Heterozygous paternal markers were inherited by putative androgenotes 10 times out of a possible 16 (Table 3).

Inheritance of MHC and SSR-PCR markers by putative androgenotes: The MHC primer pair did not produce an informative marker as it was monomorphic between the parents. Likewise, 15 of the 16 SSR primers tested did not detect parental polymorphisms. SSR primer set 29 (GOFF et al. 1992), fluorescently labeled, detected two maternal-specific and two paternal-specific markers. The paternal markers are ssr29f.153 and ssr29f.189, and the maternal markers are ssr29f.164 and ssr29f.179. The two maternal markers appear to be different loci as both markers occur in some BDP. Likewise the paternal bands are assumed to be different loci as both markers occurred in some BDP. Maternal marker ssr29f.164 was found in four out of five BDP tested and is assumed to be heterozygous. Maternal marker ssr29f.179 was found in five out of five BDP tested, and is assumed to be homozygous. Neither of these maternal markers were found in any of the four putative androgenotes. Both paternal markers were found in all four putative androgenotes.

Confirmation of fertility of a diploid androgenote: We have produced several putative androgenotes that have survived to adulthood. The androgenetic nature of a male fish (progeny of family B) that has sired hundreds of offspring was analyzed using fluorescent RAPD markers (primer 208BCF was used). Two paternal markers, which were designated as homozygous based on their occurrence in all seven BDP tested, were both found in the breeding putative male diploid androgenote. Two homozygous and one heterozygous maternal markers were not transmitted to this breeding male diploid androgenote.

Efficiency of production of androgenotes: Five crosses were made to test the percentage of normal haploid appearing embryos resulting from irradiating eggs with 10,000 R of X-rays; the range varied from 8 to 28% (Table 4). If normalized relative to the control groups, the percentages range from 10 to 37%. The first four crosses were performed in one morning and the fifth the following morning.

I/NHS (irradiated and not heat shocked) embryos, when viewed at 24 and 48 hr p.f., displayed a range of morphological phenotypes, ranging from haploid appearing with no noticeable morphological abnormalities (scored as A in Table 4, with example of one shown in Figure 2) to balls of cells that had arrested development before 24 hr. Examples of phenotypes observed more than once in embryo scored as category B (Table 4) included: developed head with diminished body and no tail, developed body and head with no tail, and body and tail with little or no head. The occurrence of certain morphological abnormalities was more common in some families than others.

When the milt for use in producing androgenotes was obtained from a fish of the SFU line, which has not been screened for recessive lethals as has the *AB line, the efficiency of production of putative haploid androgenotes (category A in Table 4) was similar to that when milt was obtained from a *AB fish.

We have scored >1200 embryos from 12 families to date that resulted from eggs irradiated with 10,000 R of X-rays and not heat shocked. We have never observed an embryo with a diploid appearance resulting from this I/NHS treatment. Data on seven of these 12 families are not presented in Table 4, as the morphological characterization of abnormal androgenotes was less thorough.

To date we have produced 44 putative diploid androgenotes; thirteen of them survived past 20 days. Production of large numbers of diploid androgenotes has not been attempted, as our rearing facility is not large.

Genetic analysis in multiple families: Using RAPD primers 208BCF and 210BCF, we surveyed a sample of putative haploid androgenotes (category A in Table 4) from four additional families (D–F) for inheritance of maternal and paternal specific markers (Table 5). Thus, 18 putative androgenotes in total were genetically ana-
TABLE 4
Production rates of androgenetic haploids

<table>
<thead>
<tr>
<th>Family</th>
<th>Male</th>
<th>Female</th>
<th>Control</th>
<th>Irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>A (%)</td>
<td>B (%)</td>
<td>C/D (%)</td>
</tr>
<tr>
<td>D</td>
<td>SFU</td>
<td>SFU</td>
<td>67</td>
<td>76</td>
</tr>
<tr>
<td>E</td>
<td>*AB</td>
<td>SFU</td>
<td>85</td>
<td>66</td>
</tr>
<tr>
<td>F</td>
<td>*AB</td>
<td>SFU</td>
<td>39</td>
<td>69</td>
</tr>
<tr>
<td>G</td>
<td>*AB</td>
<td>SFU</td>
<td>27</td>
<td>78</td>
</tr>
<tr>
<td>H</td>
<td>*AB</td>
<td>SFU</td>
<td>57</td>
<td>72</td>
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</tbody>
</table>

Eggs in the control group were held at room temperature and fertilized at same time irradiated eggs were fertilized. Milt and eggs for the five families were collected from five separate males and females. The five groups of eggs were irradiated separately at 10,000 R. Embryos were scored 2 days after fertilization based on appearance. Percentages are not normalized relative to control groups. n, sample size; AA, diploid phenotype with no morphological abnormalities apparent; A, haploid phenotype as described in Figure 2; B, haploid phenotype with noticeable morphological imperfections such as bent tail or missing part of tail; C/D, grossly abnormal embryos (classification based on WALKER and STREISINGER 1994c). In the control groups, only dead eggs and embryos that were normal diploid in appearance were observed. In the irradiated group, no embryos having a diploid appearance were observed. Data from families A–C, inclusive, are not included in this table as the progeny were not double checked by a second observer for agreement of classification into above categories.

lyzed. No maternal markers were found in any of the 18 embryos analyzed that had been irradiated with 10,000 R of X-rays.

DISCUSSION
The genetic analysis presented here, and discussed below, demonstrates the successful production of dip-

TABLE 5
Summary of genetic marker analysis for six families of androgenotes

<table>
<thead>
<tr>
<th>Family</th>
<th>Indiv.</th>
<th>Parental specific markers in family</th>
<th>Parental markers observed in putative androgenotes</th>
<th>Probability of not observing maternal markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P₀ ♂</td>
<td>P₀ ♀</td>
<td>P₀ ♂</td>
</tr>
<tr>
<td>A</td>
<td>PHA1</td>
<td>7</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>A</td>
<td>PHA2</td>
<td>7</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>A</td>
<td>PDA1</td>
<td>7</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>A</td>
<td>PDA2</td>
<td>7</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>PDA1</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>PDA1</td>
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<td>9</td>
<td>6</td>
</tr>
<tr>
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<td>PDA2</td>
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<tr>
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<td>PHA1</td>
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<td>9</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>PHA2</td>
<td>6</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>D</td>
<td>PHA1</td>
<td>1</td>
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<tr>
<td>D</td>
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<td>2</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>PHA3</td>
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<td>2</td>
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<td>PHA1</td>
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<td>7</td>
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<tr>
<td>F</td>
<td>PHA1</td>
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<td>9</td>
<td>8</td>
</tr>
<tr>
<td>F</td>
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<tr>
<td>F</td>
<td>PHA3</td>
<td>10</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>Total</td>
<td>102</td>
<td>157</td>
<td>89</td>
</tr>
</tbody>
</table>

Genescan RAPD marker analysis is summarized. Indiv., Individual androgenote analyzed; PHA, putative haploid androgenote; PDA, putative diploid androgenote. Individuals analyzed were assigned numbers within families (e.g., PHA1, indicates putative haploid androgenote number one in family indicated). Probabilities indicate the chance of not observing the maternal specific markers in a normal biparental diploid progeny. See DISCUSSION for method of calculating probabilities, and the associated assumptions. Family A is the family for which in-depth analysis was performed. Eight of the maternal markers for this family are apparently homozygous as discussed in text. PDA1 of family B, is the male diploid androgenote that we have bred. Two of the maternal markers in this family are apparently homozygous as discussed in text. It was not determined if the markers in families C–F, inclusive, were homozygous or heterozygous; thus probabilities were calculated for these families based on the conservative assumption that all markers are heterozygous in the mother. The underlying assumptions of the markers being unlinked and acting as normally segregating Mendelian markers was not tested for markers in families C–F.
genetic research and the evolutionary origin of genetic imprinting.

Confirmation of androgenetic inheritance in family A: The androgenetic nature of progeny in the one family experiment was confirmed by lack of inheritance of 12 (11 RAPD + one SSR) heterozygous maternal and six (five RAPD + one SSR) apparently homozygous, maternal DNA markers to all four androgenetic progeny tested. Although it is possible that some DNA leakage from the mother occurred, none was detected and the results strongly indicate that genomic DNA inheritance to the progeny was mostly or entirely from the male parent.

A RAPD marker was presumed homozygous in the parent if it occurred in all 12 biparental diploid progeny tested (Table 2, footnote a). The chance of a biparental diploid progeny inheriting a marker designated as homozygous, based on it being found in all 12 previously tested biparental diploid progeny, is: (1.0 × chance of marker being homozygous) + (0.5 × chance of marker being heterozygous) = (1 × 0.99975586) + (0.5 × 0.00024414) = 0.99987793. Thus, the chance of not finding one of these presumed homozygous markers in a biparental diploid progeny is 1 – 0.99987793 = 0.00012207, or ~1 in 10,000. The chance that none of the 11 heterozygous, and none of the five apparently homozygous maternal markers, being inherited by a single biparental diploid progeny can also be estimated: (0.5)11 × (0.00012207)5 = 1.3235 × 10⁻²⁸. This calculation assumes all markers are segregating as Mendelian markers and are independently assorting. Although some of the maternal heterozygous markers may be weakly linked, our analysis of random assortment of markers showed no complete linkage between any two markers. The chance that four biparental progeny would receive none of these 16 maternal RAPD markers is: (1.3235 × 10⁻²⁸)4 = 3.068 × 10⁻⁵².

The androgenetic nature of the putative androgenetic progeny is further supported by the lack of maternal SSR-PCR markers and presence of paternal SSR-PCR markers in these progeny. The chance that neither the maternal heterozygous nor the apparently homozygous SSR markers (see RESULTS) would be found in four biparental diploid progeny is 3.7 × 10⁻⁷. Combining the RAPD and SSR maternal marker data, the chance that none of the markers would be found in four biparental diploid progeny is 1.1 × 10⁻⁰⁹. This strongly suggests androgenetic inheritance.

All of the apparently homozygous, paternal RAPD markers and a proportion of the heterozygous paternal markers were inherited by all four putative androgenotes analyzed. The proportion of paternal RAPD markers inherited by the progeny is consistent (P = 0.45) with the Mendelian expectation that heterozygous markers will be inherited by half the progeny by chi-square testing for goodness of fit (ZAR 1974). Thus, it appears that androgenotes are inheriting paternal markers in a Mendelian fashion and not inheriting maternal markers.

The androgenetic nature of these fish is further supported by the phenotype of the irradiated embryos. Only severely abnormal embryos or embryos exhibiting the haploid syndrome were observed when irradiated eggs were inseminated. Following insemination of eggs irradiated with 10,000 R, in >1200 embryos observed, we have never observed the diploid phenotype (Figure 2), unless the zygotes were subsequently treated to inhibit the first mitotic division. This evidence suggests that the irradiation dose is sufficient to eliminate most or all the maternal DNA and also that the heat shock procedure is effective in restoring embryos to the normal diploid phenotype. That the irradiation dose used (10,000 R) is sufficient to prevent inheritance of maternal DNA is further supported by the coincidence of this dose with the secondary peak on a plot of survival, as a function of dosage (Figure 1), known as the Hertwig effect (HERTWIG 1911). The initial decline in survival is thought to be due to partial destruction of the maternal genome leading to aneuploidy, while further irradiation leads to complete destruction of the irradiated genome (ARAÍ et al. 1979; DON and AVTALION 1988). Although the Hertwig effect was originally observed for irradiated sperm (HERTWIG 1911), we have noted similar survival curves for irradiated zebrafish, chinook salmon (Oncorhynchus tshawytscha), and rainbow trout (O. mykiss) eggs.

In combination, the RAPD marker evidence, the SSR marker evidence, the absence of the normal diploid phenotype in the irradiated and not heat shocked group of progeny, and the observation of the Hertwig effect, provide strong evidence to support androgenetic inheritance.

Confirmation of fertility of a diploid androgenote: Genetic analysis of a putative androgenetic breeding male zebrafish, indicated it has an androgenetic genome. The chance of a BDP not inheriting two homozygous maternal (found in all seven BDP), nor one heterozygous maternal marker is 7.6 × 10⁻⁴, indicating that this breeding fish has an androgenetic genome.

Morphological appearance of haploid androgenotes: Abnormalities, viewed at 24 and 48 hr p.f., including underdeveloped heads, bodies, or tails, could be attributed to mutations carried in some individuals of the paternal line of fish, or to damage resulting from irradiation. Similar abnormalities have been observed in haploid gynogenotes produced from the *AB line of zebrafish (C. Walker, personal communication). This

[1] The large number of significant figures are included for calculation purposes only and do not indicate an exact probability as some markers, as discussed in text, may not be assorting independently to progeny.
suggests that some of the abnormalities result from background mutations in the *AB line.

**Efficiency of production of androgenotes:** The observed efficiency of production of haploid androgenotes (category A, Table 4) in five families (D–H) ranged from 8 to 28%. If categories A and B (Table 4) are combined, the production efficiency of haploid androgenotes ranged from 28 to 55%. Although we initially used milt from *AB* males because this line was screened to reduce recessive lethals, we have achieved good results with milt from the SFU line of fish which are believed to be relatively heterozygous as they originated from several pet stores and presently are not homogenous in appearance. This suggests that milt useful for producing androgenotes, does not need to be obtained from a line of fish screened for recessive lethals.

- The efficiency of production of diploid androgenotes in family A was 1.3%. To date, we have achieved success rates up to 2.1% for production of diploid androgenotes. In our facility, if fish live past the first 20 days, they usually survive through adulthood. This applies both to diploid biparental and diploid androgenotic progeny. Thus, survival was measured at day 25. At 2% efficiency, six diploid androgenotes can be expected from a batch of 300 eggs.

- Some abnormalities observed in haploid androgenotes are likely to have resulted from irradiation damage to cytoplasmic components of the oocyte. Since cytoplasmic components are known to be damaged by soft (low energy) X-rays, the efficiency of production of androgenotes might be increased by: filtering out soft (low energy) X-rays, using an X-ray machine with a higher KeV output, or using a gamma irradiation source (e.g., 60Co or 137Cs).

- **Androgenesis in other teleosts:** Attempts to produce androgenetic fishes have been reported by several groups (reviewed by Hissen et al. 1990). Putative haploid2 androgenetic embryos did not survive to the active feeding larval stage (Romashov and Belyaeva 1964; Arai et al. 1979; Parsons and Thorgaard 1984). The production and survival of diploid androgenetic salmons has been reported (Parsons and Thorgaard 1985; Scheerer et al. 1986, 1991; May et al. 1988). These fishes were reported to be androgenetic based on their being homoygous at several loci, as determined from enzyme expression assays. However, the use of DNA polymorphisms allows for direct assessment of parental alleles, irrespective of their state of expression. Thus, it provides more compelling genetic evidence for lack of maternal inheritance to androgenetic progeny.

- **Androgenesis as a genetic tool:** The production of androgenetic zebrafish has significance for investigation of several biological phenomena and provides a useful genetic tool. The process of collecting eggs and milt and irradiating and fertilizing them can be accomplished by one person in <1 hr. If heat shocking is performed, an additional 20 min is required. Tens of thousands of eggs can be irradiated simultaneously in the X-ray machine we use.

- **Male-specific meiotic recombination rates:** Knowledge of the meiotic recombination rate in each sex during gametogenesis is important for genetic studies. In humans and mice, the male meiotic crossover rate is approximately half that found in females and no crossing over occurs during meiosis in Drosophila males. Postlethwait et al. (1994) have determined the male specific cross over rate for numerous RAPD markers on all 25 zebrafish chromosomes by analyzing markers inherited by gynogenetic haploid zebrafish. The male specific cross over rates could be determined using a similar procedure, except that inheritance would be assessed in haploid androgenetic rather than haploid gynogenetic progeny. As the present RAPD map is based on female meioses, male specific markers (if any exist) would not have been observed. Thus, any new linkage group that might show up during mapping with androgenetic haploids, might be male specific, and might include sex determining genes.

- Only 94 gynogenetic haploid embryos were used to produce the zebrafish linkage map (Postlethwait et al. 1994). To produce 100 androgenotes for a linkage map based on male cross over rates would require irradiating 1000 eggs, assuming a 10% production rate of androgenetic haploids. Assuming 100 eggs/female, eggs would need to be collected from 10 females. Because >800 eggs can on occasion be collected from one female, a 28% efficiency of production would produce 224 haploid androgenotes from a single cross.

- **Sex determination:** The mechanism of sex determination in zebrafish is presently unknown (Hörstgen-Schwark 1993; Martin and McGowan 1995a), but androgenotes may provide some insight. If zebrafish have an XY-like sex determining system, those genes required on the X chromosome for survival and fecundity must also reside on the Y chromosome as male androgenotes both survive and breed. If zebrafish have an XY sex determining mechanism, then male androgenotes (YY) when bred to a normal female (XX), would result in only male (XY) progeny. Streisinger (1981) and Hörstgen-Schwark (1993) both found strongly skewed sex ratios in diploid homozygous gynogenetic progeny. Streisinger (1981) observed mainly females and Hörstgen-Schwark (1993) observed only males (two experiments: n = 9 and n = 8). If the probability of being male or female is equal, the chance of 17 progeny all being male is one in 130,000. Thus, Hörstgen-Schwark’s result is unlikely to be due to small sample size alone. There is likely to be an environmental influence that may override any genetic mechanism of...
sex determination in zebrafish, which must be considered in interpreting sex ratios of progeny from androgenotes.

Cryopreservation of allelic combinations: Androgenesis may be useful for storing and retrieving desirable combinations of certain alleles, clonal lines or wild (e.g., salmon) stocks. Zebrafish milt can be cryopreserved, (Harvey et al. 1982; Walker and Streisinger 1994a,b) but we are not aware of any reports of successful fertilization of any previously frozen teleost eggs. Frozen sperm is generally not as effective in fertilizing eggs as the milt from one fish can be used to attempt fertilization of thousands of eggs. Although we have never attempted to produce diploid androgenotes from frozen sperm, we cannot foresee any reason why it should not be possible. We are hopeful that in the future the efficiency of both fertilization using frozen sperm and efficiency of production of diploid androgenotes will improve.

Mutation screening: Androgenetic F1 haploid screens in theory might have certain advantages over gynogenetic haploid screens in mutagenesis protocols. Mutations can be induced, as for gynogenetic screens, by irradiation of sperm, eggs, or early embryos. Part of the milt obtained from the male containing the mutagenized germ-line could be used to produce androgenetic haploids for the F1 screen, and the rest frozen and used only if mutations of interest were detected. Thus, in principle, the mutagenized parent need not be retained as the mutation can be recovered from cryopreserved sperm following mutation screening.

If a haploid androgenesis screen is attempted, a background set of haploid abnormalities is expected, similar to those which are found during mutation screening using gynogenetic haploids (C. Walker, personal communication). Induced mutations can be identified by the new appearance of specific haploid abnormalities that are particular to a certain family.

Genomic imprinting: Completion of mouse embryogenesis requires both the maternal and paternal genomes because of imprinting (parent-of-origin mono-allelic expression) of essential genes in male and female gametes (McGrath and Solter 1984; Surani et al. 1984; Surani 1986; Barra and Renard 1988; Sapieza 1990; Renard et al. 1991; Gold and Pederson 1994; Ohlsson et al. 1994; Chaillet et al. 1995). This does not appear to be the case for zebrafish. Diploid homozygous gynogenotes not only complete embryogenesis, but survive to adulthood (Streisinger et al. 1981). Our results show that this is also true of diploid homozygous androgenotes. These results suggest that imprinting, in either of the parental gametic genomes, does not result in essential genes being irreversibly inactivated during a time when required for development.

While parent-of-origin (gametic) inactivation of essential genes has been ruled out in zebrafish, parent-of-origin effects on a transgene have been detected (Martin and McGowan 1995b). A decrease in methylation with maternal passage and an increase in methylation with paternal passage of a transgene in zebrafish was consistently observed. Thus, it appears that epigenetic phenomena associated with genomic imprinting occur in zebrafish and that parent-of-origin imprinting may occur in zebrafish but not for genes essential for development.

Zebrafish androgenetic haploid embryos are morphologically slightly abnormal (Figure 2) and arrest around day four. Zebrafish gynogenetic haploid embryos exhibit a typical “haploid syndrome”: they have short, stocky bodies, their eyes are incompletely formed at the ventral furrow and the brain is poorly sculptured. Cell size is often smaller in gynogenetic haploids than in diploids, as observed for melanocytes. Eventually they become edematous and die after 4–5 days (C. Walker, personal communication). Haploid androgenotes are indistinguishable in appearance from haploid gynogenotes, suggesting that the abnormalities are not due to parent-of-origin, but may be dependent on gene dosage.

We believe that this is the first report of the production of a viable and fertile androgenetic diploid vertebrate in which the extent of elimination of the maternal genome has been assessed by the use of DNA markers. There have been other reports of production of fertile androgenetic and gynogenetic adult teleost fishes (reviewed by Ihssen et al. 1990) and amphibians (e.g., Gillespie and Armstrong 1981). Collectively, these reports indicate that the failure of androgenesis and gynogenesis reported for some mammals (McGrath and Solter 1984; Surani et al. 1984; Surani 1986; Gold and Pederson 1994) is not characteristic of vertebrates in general. Thus, genomic imprinting of genes essential for development may be a specialized phenomenon that arose during mammalian evolution.

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