Half-Tetrad Analysis in Zebrafish: Mapping the *ros* Mutation and the Centromere of Linkage Group I

Stephen L. Johnson, Dana Africa, Sally Horne and John H. Postlethwait

Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403

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ABSTRACT

Analysis of meiotic tetrads is routinely used to determine genetic linkage in various fungi. Here we apply tetrad analysis to the study of genetic linkage in a vertebrate. The half-tetrad genotypes of gynogenetic diploid zebrafish produced by early-pressure (EP) treatment were used to investigate the linkage relationships of two recessive pigment pattern mutations, *leopard* (*leo*) and *rose* (*ros*). The results showed that *ros* is tightly linked to its centromere and *leo* maps 31 cM from its centromere. Analysis of half-tetrads segregating for *ros* and *leo* in repulsion revealed no homozygous *ros* individuals among 32 homozygous *leo* half-tetrads—*i.e.*, a parental ditype (PD) to nonparental ditype (NPD) ratio of 32:0. This result shows that *ros* is linked to *leo*, a mutation previously mapped to Linkage Group I. Investigation of PCR-based DNA polymorphisms on Linkage Group I confirmed the location of *ros* near the centromere of this linkage group. We propose an efficient, generally useful method to assign new mutations to a linkage group in zebrafish by determining which of 25 polymerase chain reaction (PCR)-based centromere markers shows a significant excess of PD to NPD in half-tetrad fish.

HUNDREDS of new mutations have recently been isolated that alter the development and physiology of the zebrafish *Danio rerio* (KIMMEL 1989; DRIEVER *et al.* 1994; MULLINS *et al.* 1994). Genetic analysis of these mutations and their molecular isolation by either chromosome walking (WICKING and WILLIAMSON 1991) or the testing of candidate genes (EPSTEIN *et al.* 1991) urgently requires efficient methods to map them (CONCORDET and INGHAM 1994). To help accomplish this goal, we have coupled the haploid genetics of zebrafish (STREISINGER *et al.* 1981, 1986) with the polymerase chain reaction (PCR, WILLIAMS *et al.* 1990; MICHELMORE *et al.* 1991) to identify DNA polymorphisms between standard strains of zebrafish and construct a genetic map of the ~2600 cM zebrafish genome (JOHNSON *et al.* 1994; POSTLETHWAIT *et al.* 1994). This has led to the identification of molecular genetic markers closely linked to both embryonic lethal and visible mutations (POSTLETHWAIT *et al.* 1994).

While applying haploid genetics to zebrafish greatly simplifies the mapping of embryonic lethal mutations, we have asked whether gene mapping could be improved by adopting additional methods of microbial genetics. Analysis of tetrads, the four products of a single meiotic division, has long been the method of choice for determining linkage relationships in Neurospora, yeasts, Ustilago, and other fungi (PERKINS 1949; MORTIMER and SCHILD 1981). When two genes are unlinked, the frequency of the parental ditype class of tetrads (PD) is equal to the frequency of the nonparental ditype tetrads (NPD). When loci are linked, even loosely, the PDs exceed the NPDs, the latter class arising by four-strand double crossovers (see Figure 1).

Despite its power, tetrad analysis has not been extensively used in plants and animals. This is because only one of the four haploid products generally survives in female meiosis, and the four haploid products in male meiosis are seldom bundled together. Thus, tetrad analysis can be applied to plants and animals only in special situations, for example, by exploiting mutations that block pollen separation in *Arabidopsis thaliana* (PREUSS *et al.* 1994). In other cases, half-tetrads, the sister chromatid products of meiosis I, are available and can provide much of the same information as full tetrads. Examples include specially designed compound chromosomes in Drosophila (BEADLE and EMERSON 1935; BALDWIN and CHOVNICK 1967), impaternate daughters of the wasp Habrobracon (SPEICHER 1934), and spontaneous suppression of the second meiotic division in some ovarian teratomas in mice and humans (EPPIG and EICHER 1983; CHAKRAVARTI *et al.* 1989).

In contrast to most animals, in zebrafish, meiotic half-tetrads can be produced routinely from any genotype constructed. STREISINGER *et al.* (1983) showed that blocking the second meiotic division with high pressure (early-pressure parthenogenesis, EP) leads to gynogenetic diploid ova bearing half-tetrad chromosomes. When these EP ova are fertilized with sperm that has been inactivated by UV light, the EP half-tetrads develop as gynogenetic diploids. When no lethal mutations are involved, these generally survive to normal adult stages. Moreover, the fraction of EP half-tetrads

Corresponding author: Stephen L. Johnson, Institute of Neuroscience, University of Oregon, Eugene, OR 97403.
E-mail: sjohnson@uoneuro.uoregon.edu

that are homozygous for loci that were heterozygous in the mother is a function of the distance between the locus and its centromere. The interval, in centimorgans (cM), between a mutation and its centromere can be estimated by subtracting twice the percentage of homozygous mutants from 100 and dividing by two (STREISINGER *et al.* 1986). This mapping function, which assumes absolute interference, seems to work well for small gene-centromere distances. Thus, recessive mutations that appear homozygous in nearly half of the EP progeny of a heterozygous female are relatively close to the centromere—for example, *sparse* heterozygotes give 38% homozygous mutant EP progeny, predicting a distance of 12 cM to its centromere (STREISINGER *et al.* 1986). This prediction has since been confirmed by mapping the *spa* mutation (POSTLETHWAIT *et al.* 1994) and its centromere (S. JOHNSON and S. HORNE, unpublished results). By contrast, recessive mutations that appear only infrequently as homozygotes among EP progeny from heterozygous females, such as *golden* (*gol*)—that results in 5% *gol* homozygotes in the EP progeny of heterozygous females—should map far from the centromere. The small proportion of *gol* mutant progeny among EP progeny is indicative of strong chiasma interference in zebrafish meiosis (STREISINGER *et al.* 1986). Further understanding of properties of interference in zebrafish may require the mapping of both the *gol* gene and its corresponding centromere, using methods described here. In view of this interference, STREISINGER *et al.* (1986) proposed that the lack of doubly homozygous half-tetrads from females heterozygous for two recessive mutations in repulsion would be a sensitive test for linkage in zebrafish.

We pursued STREISINGER's suggestion while investigating linkage relationships of two recessive adult pigment pattern mutations, *leopard* (*leo*) a mutation that we previously mapped to Linkage Group I (POSTLETHWAIT *et al.* 1994), and *rose* (*ros*, JOHNSON *et al.*, 1995), a previously unmapped mutation. Half-tetrad analysis demonstrated that *ros* is tightly linked to its centromere and that *leo* is 31 cM from its centromere. Half-tetrads segregating both *ros* and *leo* failed to generate double mutants, indicating that the two genes are linked to the same centromere. Conventional linkage analysis using DNA polymorphisms on Linkage Group I (POSTLETHWAIT *et al.* 1994; S. JOHNSON, unpublished results) confirmed the genetic linkage of *leo* and *ros*.

In light of these results, we suggest a generally useful and efficient method to assign a mutation to a linkage group by assessing segregation of mutations with respect to segregation of PCR-based centromere-linked markers in the homozygous mutant segregants in half-tetrad zebrafish. In EP half-tetrads, homozygous mutants can arise either when there have been no crossovers between the mutant locus and its centromere, a two-strand double crossover (both resulting in PD half-tetrads) or a four-strand double crossover (resulting in a NPD half-tetrad; see Figure 1 for some examples of half-tetrad types that can arise from various meiotic configurations). Because four-strand double crossovers are likely to be rare, the linked centromere should be identified by an excess of PD:NPD half-tetrads. In contrast, independent segregation of unlinked centromeres should show PD:NPD ratios of 1:1.

MATERIALS AND METHODS

Stocks and mutations: The DAR (Darjeeling) strain was isolated from fish captured in the wild in 1987 and subsequently brought to Eugene. An inbred and isocentromeric isolate of the DAR strain, SJD, was isolated from a partially inbred population of DAR by subjecting it to two sequential generations of brother-sister mating, followed by two sequential generations of EP parthenogenetic reproduction. This breeding scheme results in homozygosis for many loci, including loci near the centromere. As a result of two sequential generations of EP, SJD is presumably isocentromeric for all linkage groups. Preliminary results suggest that >80% of random amplified polymorphic DNA loci (RAPDs, WILLIAMS *et al.* 1990) are homozygous in this line. The outbred DAR strain has not been maintained. C32 is a clonal, presumably homozygous, derivative of STREISINGER's outbred population AB (STREISINGER *et al.* 1981).

ros (*ros*^{b140}) and *leopard* (*leo*^{tu1}) mutations have been described (JOHNSON *et al.* 1995). In homozygous *leo* fish, dermal melanocytes underlying silver iridophores are clustered in spots rather than stripes (KIRSCHBAUM 1976; JOHNSON *et al.* 1995). *ros* is a previously unmapped mutation that deletes iridophores and about half the dermal melanocytes. The melanocytes that persist in *ros*, however, are arranged in distinct stripes (JOHNSON *et al.* 1995). Fish doubly homozygous for *ros* and *leo* are easily distinguished from either single mutant because iridophores are absent, as in *ros*, but the melanocytes that persist are dispersed throughout the flank, having failed to migrate into the stripe positions, an activity that requires the function of the *leo* gene (JOHNSON *et al.* 1995).

The *leo* mutation was obtained from Dr. NUSSLEIN-VOLLHARD on an uncharacterized genetic background and subsequently crossed into the C32 clonal background (three outcrosses) before experiments described here. The *ros* mutation arose spontaneously and has been maintained in a C32/AB mixed genetic background.

Crosses: Females were generated for EP reproduction by crossing a *ros*/*ros*, *leo*/*leo*$^{+}$ female to a *ros*$^{+}$/*ros*$^{+}$, *leo*/*leo* male. Mature *ros*/*ros*$^{+}$, *leo*/*leo*$^{+}$ and *ros*/*ros*$^{+}$, *leo*/*leo* females were subjected to EP parthenogenetic reproduction as described (STREISINGER *et al.* 1981; WESTERFIELD 1993). To map *ros*, a single *ros*/*ros* female was crossed to a single DAR male, and progeny were reared to maturity. A single heterozygous hybrid female was then backcrossed to a *ros*/*ros* male and the backcross progeny were reared until the *ros* phenotype could be scored and fins amputated for DNA extraction. Analysis of segregants from C32 × DAR F1s was used to delimit the map position of the Linkage Group I centromere (in this case, the DAR fish used in this cross was among the progeny of the first round of EP used to obtain isocentromeric SJD line described above). Haploid segregants from C32 × SJD F1s were typed with RAPD markers to establish a reference mapping cross based on inbred zebrafish lines.

Mapping: RAPD markers closely linked to *ros* were identified by bulked segregant analysis as described (POSTLETHWAIT *et al.* 1994). Progeny from a [(*ros* × DAR) × *ros*] backcross were reared to adult stages, scored for the *ros* phenotype, and small portions of fins were excised (fin clips) from anesthetized fish

TABLE 1

Segregation of *ros* and *leo* in repulsion in EP half-terads

	Phenotypes in EP half tetrads				Fraction	
	ros$^+$		*ros*$^-$			
Clutch	*leo*$^+$	*leo*$^-$	*leo*$^+$	*leo*$^-$	*ros* homozygotes	*leo* homozygotes
1	22	13	41	0	0.54	0.17
2	7	2	9	0	0.50	0.11
3	6	4	11	0	0.52	0.19
4	6	3	7	0	0.44	0.19
5	14	10	17	0	0.41	0.24
Total	55	32	85	0	0.49	0.19
6–11[a]		136		144	0.51	
					0.51	0.19

Females heterozygous for *ros* and *leo* in repulsion (1–5) were subjected to EP parthenogenetic reproduction and progeny were reared to maturity and scored for *ros,leo* and *ros,leo* phenotypes. Estimated distance to centromere for *ros*, 0 cM; *leo*, 31 cM.

[a] EP progeny of six *ros* heterozygous, *leo* homozygous females.

for DNA extraction and PCR analysis. In bulked segregant screens, DNA from 20 *ros/ros* mutant segregants was pooled, and DNA from 20 wild-type (*ros/ros*$^+$) segregants was pooled. Pairwise PCR reactions with each of several hundred RAPD primers were screened for amplification products specific to the wild-type pool. Candidate markers were those amplified from DNA pools of *ros/ros*$^+$ fish and absent from from the pool of DNAs from the *ros/ros* fish. Candidate markers were then scored for potential linkage to *ros* in a set of DNAs from individuals that did not contribute to the bulk. Those that indicated linkage to *ros* were then assessed on the entire set of 83 backcross progeny. By these means, three markers were identified that mapped within 10 cM of *ros*, including *14AD.1600*, reported here. The map of RAPD markers on Linkage Group I (POSTLETHWAIT *et al.* 1994) was extended by genotyping the haploid progeny of (C32 × SJD) hybrids. Gene-centromere distances (in centimorgans) were determined according to STREISINGER *et al.* (1986): $50[1 - (2m/N)]$, where m is the number of homozygous recessive half-tetrads and N is the total number of half-tetrads examined.

RESULTS

Linkage of *ros* to its centromere: To determine the genetic distance between *ros* and its centromere, we constructed females that were heterozygous for *ros* (and either heterozygous or homozygous for *leo*, see below). From these females, we collected EP parthenogenetic offspring for half-tetrad analysis. Among 452 EP progeny of *ros/ros*$^+$ females, 229 were homozygous for *ros* (51%, see Table 1). Because this result is inconsistent with appreciable recombination between the mutation and its centromere, we conclude that *ros* is tightly linked to its centromere.

Linkage of *leo* to its centromere: The gene-centromere distance for *leo* was determined in the half-tetrad offspring of females that were heterozygous for both *ros* and *leo*. Among 172 EP offspring of *ros/ros*$^+$, *leo/leo*$^+$ in repulsion females, 32 were homozygous *leo* (19%), showing that *leo* lies 31 cM $\{50[1 - (2 \times 32)/172]\}$ from its centromere (see Table 1) assuming strong interference.

Linkage relationships of *ros* and *leo*: To determine whether *ros* and *leo* are linked, we examined 172 EP progeny from clutches segregating both mutations. We looked at the data in two different ways. First, if *ros* and *leo* were segregating independently, we should find double mutants in a frequency reflecting the product of the two individual mutant frequencies. Because *leo* homozygotes are 19% of all EP half-tetrads, and *ros* homozygotes are 51% of EP half-tetrads, then we should expect ~10%, or 17 double mutants in the 176 EP half-tetrads if *leo* and *ros* are unlinked. Instead, we found no *ros, leo* double mutants among these EP animals, demonstrating that the two mutations are linked.

In the other way of looking at the data, we determined the ratio of parental ditypes to NPD among the EP progeny of the cross segregating both mutations (Table 1). If the mutations are unlinked, then the NPD class (twice the phenotypically double mutant animals) should be as frequent as the PD class (twice the homozygotes for the distal-most marker, in this case *leo*). If the mutations are linked, then the PD class should outnumber the NPD class, which arises only from infrequent four-strand double recombinant meioses (Figure 1). The results showed a ratio of PD to NPD of 32:0, ruling out the unlinked hypothesis. Because *leo* was previously mapped to Linkage Group I, *ros* must also map to Linkage Group I. Because EP data indicate that *leo* is 31 cM from the centromere, and *ros* is very close to the same centromere, *ros* and *leo* must map ~31 cM from each other.

Identifying DNA polymorphisms closely linked to *ros*: In an independent effort to map the *ros* mutation, bulked segregant screens were used to identify arbitrary decameric PCR primers that amplified markers closely linked to *ros* (MICHELMORE *et al.* 1991; POSTLETHWAIT *et al.* 1994; see MATERIALS AND METHODS). One marker, *14AD.1600* was amplified in all 42 *ros/ros*$^+$ animals, and

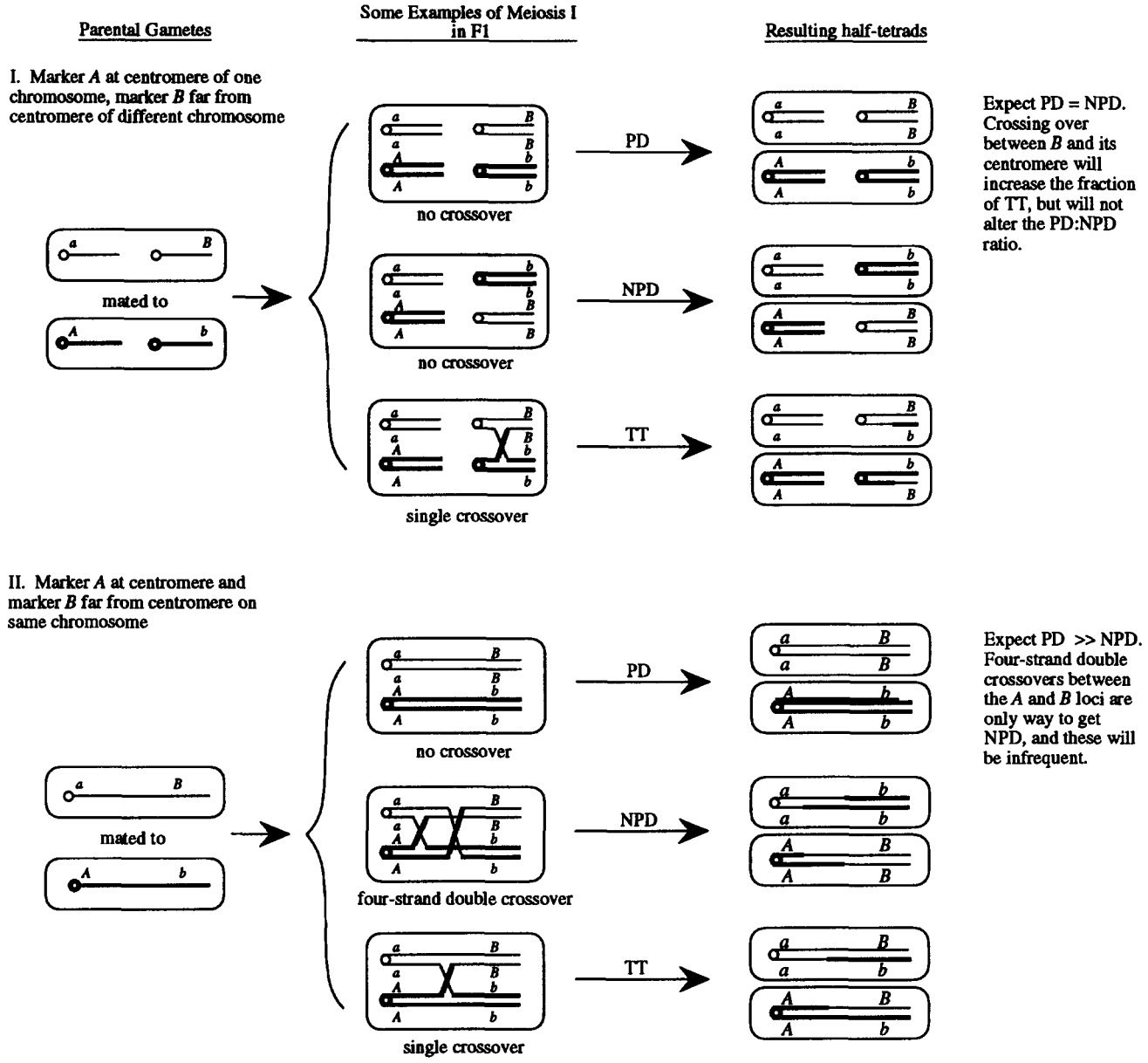

FIGURE 1.—Schematic representation of meiosis leading to parental ditype (PD), nonparental ditype (NPD) and tetratype (TT) half-tetrads.

not amplified in all 41 *ros/ros* individuals from the backcross (see Figure 2). The absence of recombinants in 83 individuals indicates that *14AD.1600* is within 1.2 cM (95% confidence interval) of *ros*.

Placing *ros* on the RAPD map for Linkage Group I: We wished to map *ros* with respect to the previously described RAPD markers for Linkage Group I. Because some of the DAR-derived Linkage Group I markers were not segregating in the [(*ros* × DAR) × *ros*] backcross described above (presumably reflecting the outbred nature of the early DAR populations), we reasoned that we could achieve the same goal by assessing segregation of the *ros*-linked marker *14AD.1600* in haploid embryos from a C32 × SJD hybrid female that had previously been genotyped for a large number of Linkage Group I markers. We refer to crosses that are genotyped for large numbers of markers as reference crosses. Because the *ros*-linked marker *14AD.1600* segregated in the C32 × SJD reference cross, standard three-point cross analysis could be used to localize the position of *14AD.1600* (and thus *ros*) with respect to other Linkage Group I RAPD markers. This analysis indicated that *14AD.1600* is flanked by *8F.1400* and *17AE.1450*, with *14AD.1600* mapping 1.5 cM from the former and 2.2 cM from the latter flanking marker. Because *ros* maps within 1.2 cM (95% confidence interval) of *14AD.1600*, which is less than the interval observed between *14AD.1600* and the flanking markers, we propose the marker order is *8F.1400-(14AD.1600/ros)-17AE.1450* (see Figure 3). The flanking markers were not segregating in the [(*ros* × DAR) ×

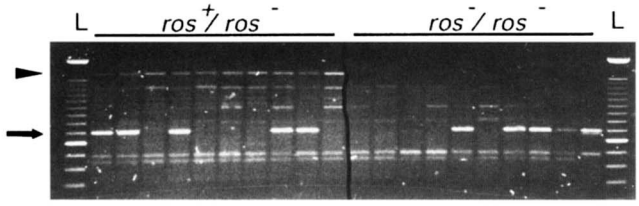

FIGURE 2.—RAPD marker *14AD.1600* is tightly linked to *ros*. DNA was prepared from fin clips of mature progeny from a [(*ros* × DAR) × *ros*] backcross and scored for segregation of RAPD markers. Shown here are PCR amplification products from the RAPD primer 14AD (Operon primer OAD14) on 10 *ros* and 10 wild-type progeny separated on a 1% agarose gel. Marker *14AD.1600* (arrowhead) segregates exclusively with the wild-type allele of the *ros* locus (0 recombinants in 83 animals scored). Other markers, such as *14AD.700* (arrow), are shown segregating independently of the *ros* locus. PCR reactions are flanked by 100-bp ladder (Gibco-BRL).

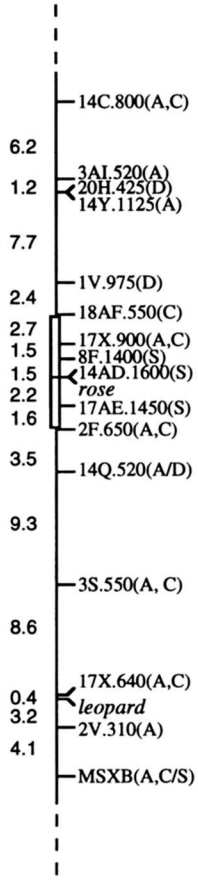

FIGURE 3.—Map of the *ros* region of Linkage Group I. Interval distances (left) are shown in centimorgans. Markers are shown on the right. Strains that carry the markers are shown in parenthesis (A, AB; C, C32; D, DAR; S, SJD). Strain designations separated by a coma indicate that both strains share the same allele. Strain designations separated by a slash indicate codominant alleles (different gel mobilities). The open rectangle indicates possible extent of centromere. All markers were described previously POSTLETHWAIT *et al.* 1994) except *14AD.1600* (this report) and *18AF.550* and *8F.1400,* which were discovered in the course of genotyping the C32 × SJD reference mapping cross.

ros] backcross (not shown), so we cannot rule out the possibility that *ros* actually lies distal of *8F.1400* or *17AE.1450* from *14AD.1600.*

Mapping the centromere of Linkage Group I with RAPD markers: We were interested in whether we could use RAPD markers to delimit the position of the centromere of Linkage Group I. We had concluded from half-tetrad studies of crosses segregating *ros* and *leo* that *ros* lies near the centromere of Linkage Group I. This conclusion assumes that there are no factors such as meiotic drive or semilethal alleles that would diminish recovery of the ros^+ chromosome among EP animals and thus increase the proportion of *ros/ros* half-tetrads. If *ros* lies near the centromere of Linkage Group I, the nearby RAPD marker *14AD.1600* should also map near the centromere. As an alternative method for mapping the centromere of Linkage Group I that lacks these biases, we analyzed 42 half-tetrad progeny from a C32 × DAR hybrid female for first or second meiotic division segregation of four RAPD markers, in addition to *14AD.1600.*

Centromeres (and their nearby markers) will always experience first division segregation, whereas markers distant from the centromere will often exhibit second division segregation. An analysis of markers distributed along Linkage Group I in half-tetrads should identify those that always or nearly always undergo first division segregation (homozygous in EP animals) and those that sometimes experience second division segregation (sometimes heterozygous in EP animals). The map position of the centromere can then be delimited by excluding chromosomal regions that are sometimes heterozygous for markers in EP half-tetrads.

Heterozygosity of markers can be detected directly with codominant markers, but must be inferred from phenotypes of flanking markers in the case of dominant markers, such as most RAPD markers. Consider, for example, how we determine the genotype of half-tetrads segregating for the five loci shown in Figure 4. The frequent (16/42) class I tetrads in Figure 4 show the allelic arrangement of the DAR parent in our cross: the recessive alleles of RAPD markers (the absence of a PCR product) for *17X.900, 18AF.550* and *2F.650* are in coupling. The reciprocal configuration for the C32 alleles contribute to the second parental class of half-tetrads (class VI).

To localize the centromere, consider the three loci with recessive alleles on the C32 chromosome in class II in Figure 4 and ignore for now the loci present as dominant alleles on that chromosome (*8F.1400* and *14AD.1600*). In the three class II half-tetrads, the recessive phenotype of the two loci on the right (*17X.900* and *2F.650*) shows that these half-tetrads are homozygous recessive. In class II half-tetrads, however, amplification of the dominant allele from the left marker *18AF.550* shows that crossing over must have occurred between the left and middle marker (*18AF.550* and *17X.900*) in

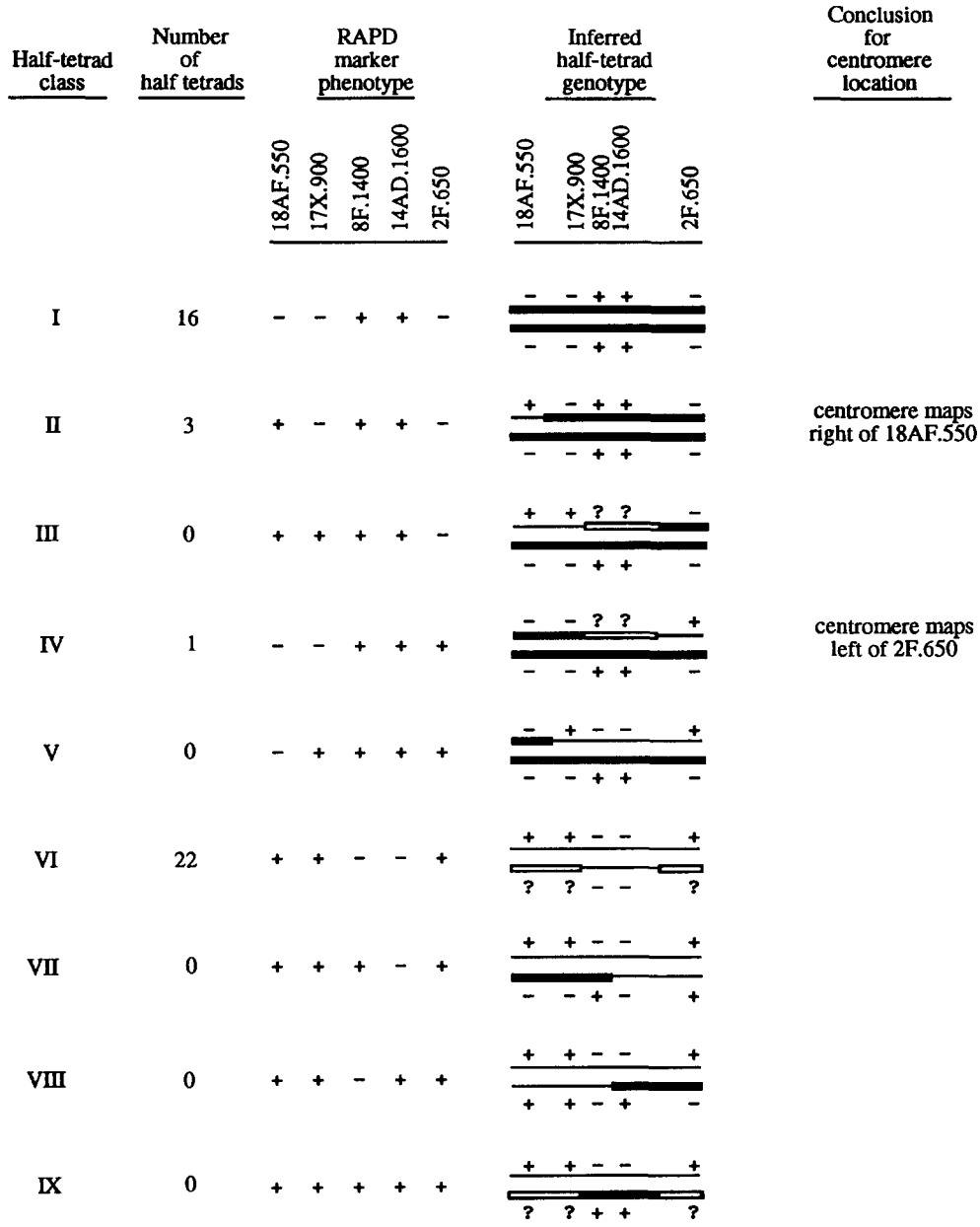

FIGURE 4.—Half-tetrad analysis of segregation of RAPD markers near the centromere of linkage group I. Forty-two half-tetrads from a C32 × DAR hybrid were analyzed for linkage group I markers (shown in order from left to right). The second column indicates number of half-tetrads of each phenotype indicated in column three. Column four shows inferred genotype corresponding to each possible phenotype. Thin or thick lines indicate the two parental chromosomes. Parental half-tetrads are contained in class I (DAR) and class VI (C32). In some classes the genotype at some loci is ambiguous, due to the dominant nature of RAPD markers. For these, ambiguity at a particular locus is indicated by an open line (classes III, IV, VI and IX).

the meioses leading to these tetrads. A single crossover would result in half-tetrads that are heterozygous at the *18AF.550* locus, whereas a double crossover, expected to be rare in these short intervals, would be required if class II half-tetrads were homozygous dominant for *18AF.550*. Therefore, in class II half-tetrads, we may infer second division segregation of the *18AF.550* locus. Because centromeres segregate in the first meiotic division, the inferred heterozygosis of *18AF.550* suggests that the centromere for Linkage Group I maps to the right of this locus, within regions that show first division segregation in class II tetrads.

Similar reasoning leads us to conclude that the right locus *2F.650* is heterozygous in class IV half-tetrads, indicating a crossover between *2F.650* and *17X.900*. Thus, analysis of this half-tetrad indicates that the centromere lies to the left of *2F.650*. Because heterozygous and homozygous states for the dominant alleles of *8F.1400* and *14AD.1600* cannot be determined in class IV half-tetrads, the crossover in class IV half-tetrads cannot be further localized with respect to *8F.1400* and *14AD.1600*, information that might have led to further delimitation of the possible extent of the centromere. Taken together, these data indicate that the centromere for Linkage

Group I lies in a 9.5-cM region between *18AF.550* and *2F.650*. This is an independent corroboration of our previous finding (Table 1) that the centromere of Linkage Group I is close to the *ros* locus, which itself is within 1.2 cM of *14AD.1600*.

DISCUSSION

These results confirm the utility of half-tetrad analysis in establishing linkage relationships in a vertebrate. A search for double mutants in EP half-tetrads segregating both *ros* and *leo*, a marker on Linkage Group I, indicated that these two genes are linked, with *ros* near the centromere and *leo* ~31 cM away. This is the first instance in a vertebrate in which half-tetrad analysis was used to initially assign a mutation to its linkage group.

The map location of *ros* determined by half-tetrad analysis was substantiated by conventional linkage studies. Bulked segregant screens for markers linked to *ros* identified a closely linked RAPD marker. Scoring the *ros*-linked marker on haploids from a cross that had previously been genotyped for a large number of markers, including those for Linkage Group I, permitted a more precise localization of the *ros* mutation. The placement of the *ros*-linked marker, and thus *ros*, on the recombination map suggests a *ros-leo* distance similar to that suggested by EP half-tetrads. Because *17AE.1450* lies 2.2 cM opposite of *ros* (with respect to *leo*; this study) and *17X.640* lies 0.4 cM opposite of *leo* (with resepect to *ros*; S. JOHNSON, unpublished results), adding these distances to the *17AE.1450* to *17X.640* interval of 23 cM suggests a *ros-leo* interval of 25.6 cM, similar to the 31 cM predicted by EP half-tetrads.

A significant advantage of gene mapping in half-tetrads is the ability to determine the location of the centromere with respect to molecular markers in addition to the location of the gene under study. We used two methods to identify the centromere of Linkage Group I. EP half-tetrads segregating *ros* positioned the centromere very close to *ros*. Subsequent placement of *ros* on the recombination map allowed us to use RAPD markers and first- *vs.* second-division segregation to delimit the centromere to a 9.5-cM region including the *ros* locus.

We are interested in improving methods for mapping mutations in zebrafish. The method that we currently use for initial linkage determination—bulked segregant screens for closely linked RAPD markers—usually establishes map location to within a few centimorgans with ~1000 PCR reactions. In this method, pools of DNA from 20 mutant or 20 wild-type segregants are screened for amplification of PCR products specific to one or the other pool using arbitrary decameric primers (when using diploid backcrosses instead of haploids, only amplification products specific to the dominant allele-containing pool are found). Primers that show amplification products specific to one or the other of these pools are then rescreened for specific amplification in a second set of pools constructed from a different set of mutant and wild-type segregants. Those RAPD markers that repeatedly show specific amplification from either mutant or wild-type pools are then scored on individual animals—12 mutant and 12 wild type—to test for *bona fide* linkage. Finally, linked markers identified by these methods must be related to the recombination map, usually by scoring a reference cross for segregation of the marker. We expect an average of three to five markers within 10 cM of the mutation when 300 decameric primers are assessed in haploid-based bulked segregant screens (S. JOHNSON and S. HORNE, unpublished results). All together, such efforts to define the initial estimate of map position for a mutation may require in excess of 1000 PCR reactions.

Other strategies to assess initial linkage relationships involve exploiting situations in which crossing over is suppressed—for example, in Drosophila males (MORGAN 1912) or in *spo11* mutant Saccharomyces (KLAPHOLZ and ESPOSITO 1983). In these cases, one can determine the appropriate linkage group by scoring relatively few individuals for both the mutation of interest and a single marker on each linkage group. EP half-tetrads may present a similar opportunity to identify nonrecombinant or reduced recombination individuals that can be used to assign mutations to linkage groups in zebrafish. Homozygous mutants in EP half-tetrad progeny of heterozygous females may arise either when no crossovers, two-strand double crossovers, or four-strand double crossovers have occurred between a mutant locus and its centromere. When the homozygous mutant EP segregants are assessed for segregation of markers near the linked centromere, the noncrossover class and the two-strand double class will appear as parental ditype (PD) half-tetrads, while the four-strand double crossovers will appear as nonparental ditype (NPD) half-tetrads (see Figure 1). Because such PD half-tetrads will exceed NPD half-tetrads, even when the mutation is loosely linked to its centromere, assessment of PD:NPD ratio for a mutation and each of the centromeres in EP half-tetrads should identify the appropriate linkage group. Independent segregation of centromeres unlinked to the mutations should reveal PD:NPD ratios of 1:1, while the appropriate linked centromere should show an excess of PD half-tetrads. The potential success of such a strategy is illustrated by the finding that among 172 EP half-tetrads segregating both *ros* (tightly linked to its centromere) and *leo*, none of the 32 *leo* homozygotes were also homozygous for *ros*. Thus, these results indicated a PD:NPD ratio of 32:0, providing the first evidence that *ros* must map to the same linkage group as *leo* (Linkage Group I).

Whether an absolute exclusion of NPD half-tetrads between a mutant locus and its centromere, such as that observed for the *leo* locus, will be observed for all

mutations is not clear. The low fraction of *gol* homozygotes in EP progeny of heterozygous *gol* females has raised the possibility that chiasma interference in zebrafish is absolute—that is, that double crossovers (including four-strand double crossovers that result in NPD half-tetrads) do not occur between a locus and its centromere in zebrafish meiosis.

While chiasma interference in zebrafish may be strong, double crossovers do occur on individual chromosome arms. In a situation where interference is absolute, there is no more than one crossover per tetrad per chromosome arm. If such were the case in zebrafish, then no chromosome arm could exceed 50 cM and no linkage group could exceed 100 cM. Results show, however, that nine of the linkage groups reported exceed 100 cM, including 122 cM for Linkage Group I and 146 cM for the largest, Linkage Group III (POSTLETHWAIT *et al.* 1994). Moreover, analysis of individual chromosomes in the data set that led to the recombination map reveals a number of chromosomes with three or more crossovers (S. JOHNSON, unpublished results), indicating that some meioses have more than one crossover per chromosome arm. Now that the centromere position of Linkage Group I is known, we can search for direct evidence for multiple crossovers per chromosome arm on Linkage Group I.

The results reported here suggest an efficient method to assign recessive mutations to linkage groups by analyzing only a few homozygous mutant segregants from appropriate EP crosses. Mutant-bearing animals could be crossed to an appropriate polymorphic mapping strain such as SJD to produce heterozygotes. After EP reproduction of heterozygous fish, DNA should be isolated from homozygous mutant segregants. Subsequent genotyping of the segregants with PCR-based markers tightly linked to centromeres for each of the 25 chromosomes should reliably identify the appropriate linkage group, based on an excess of PD over NPD half-tetrads. Our experience with *leo* and *ros* indicate that this method can be used to map mutations onto linkage groups that lie within 30 cM of their centromeres. We suggest that this method can also be used to identify linkage group assignments for mutations greater than 30 cM from the centromere. Strong interference in zebrafish meiosis may provide for high PD:NPD ratios for loci that are more distant. Even in the absence of interference, at 100 cM (the approximate length of the longest zebrafish chromosome arm), a PD: NPD ratio of 2.2:1 is expected (PERKINS 1953). Thus, markers distant from their centromere should still be detected from analysis of PD:NPD ratios of relatively few mutant segregants.

We suggest that analysis of segregation of PCR-based markers for each centromere in 10–12 homozygous mutant EP segregants, together with DNA from each of the parents as positive and negative control for amplification of the marker, should allow for linkage group assignment of most mutations. To screen 25 centromeres for potential linkage to the mutation of interest would thus be achieved in only 300 PCR reactions, a significant improvement over the ~1000 PCR reactions that are usually performed in bulked segregant screens to identify markers linked to mutations. An additional 200 reactions or so should localize the mutation to a specific location on a chromosome arm. Moreover, such methods will be equally applicable to analysis of mutations with embryonic or adult phenotypes. Preliminary mapping studies on *jaguar* (*jag*), a previously undescribed mutation that affects development of adult pigment stripes (S. JOHNSON, unpublished results), support this suggestion. Bulked segregant screens on the progeny of a [(*jag* × SJD) × *jag*] backcross failed to detect *jag*-linked markers (employing ~1200 PCR reactions). When we screened homozygous EP segregants from *jag* heterozygous females with 24 different RAPD primers (chosen either because the primer amplified markers that we had previously determined to be centromere linked, or because the primer amplified markers that are distributed along linkage groups for which centromeres had not yet been mapped), we detected a PD:NPD ratio of 12:0 for markers on Linkage Group 15 (not shown), indicating that *jag* is on Linkage Group 15 ($\chi^2 = 6$, $P > 0.98$).

General use of EP half-tetrad analysis to determine linkage groups for zebrafish mutations requires the availability of PCR-based centromere-linked markers for each of the 25 zebrafish chromosomes, such as the *14AD.1600* marker for the centromere of Linkage Group I. The identification of markers for each of the centromeres to facilitate centromere-linkage screens for linkage group assignment of mutations is currently well underway.

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