Centromere-Linkage Analysis and Consolidation of the Zebrafish Genetic Map

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ABSTRACT

The ease of isolating mutations in zebrafish will contribute to an understanding of a variety of processes common to all vertebrates. To facilitate genetic analysis of such mutations, we have identified DNA polymorphisms closely linked to each of the 25 centromeres of zebrafish, placed centromeres on the linkage map, increased the number of mapped PCR-based markers to 652, and consolidated the number of linkage groups to the number of chromosomes. This work makes possible centromere-linkage analysis, a novel, rapid method to assign mutations to a specific linkage group using half-tetrads.

RECENT studies have uncovered hundreds of mutations that disrupt specific events in zebrafish development (KIMMEL 1989; MULLINS and NUSSLEIN-VOLHARD 1993; DRIEVER *et al.* 1994; HENION *et al.* 1995; JOHNSON and WESTON 1995; JOHNSON *et al.* 1995b) Because the mechanisms of morphogenesis, organogenesis, and pattern formation are broadly shared among vertebrates (KIMMEL 1989), most of these mutations will identify the functions of genes whose homologues are involved in the development of humans and other mammals (CONCORDET and INGHAM 1994; KAHN 1994). To understand fully the processes disrupted by these mutations, precise phenotypic analysis afforded by optically clear, externally developing zebrafish embryos must be coupled to the molecular isolation of the gene each mutation disrupts. In the candidate gene approach for cloning a mutated locus, the genetic location of a mutation may identify nearby candidates for the mutated gene (TALBOT *et al.* 1995). Alternatively, in the positional cloning approach, the identification of DNA polymorphisms tightly linked to the mutant locus can serve as entry points for a chromosome walk (WICKING and WILLIAMSON 1991; KINGSLEY *et al.* 1992). These strategies are both facilitated by an extensive genetic linkage map.

Construction of the zebrafish linkage map has just begun—before 1994, no two loci had been shown to be linked in the zebrafish genome. At that time, we reported a linkage map based on 425 genetic markers distributed among 29 linkage groups (LGs) (POSTLETHWAIT *et al.* 1994). Because the haploid chromosome number is 25 (ENDO and INGALLS 1968; DAGA *et al.* 1996), the initial map had four more linkage groups than chromosomes, and hence contained at least four gaps. A major goal of the current work was to fill these gaps in the linkage map so that the number of linkage groups would equal the number of chromosomes. Because each chromosome has a single centromere, we focused on localizing centromeres on the genetic map.

Centromeres are attachment sites for spindle microtubules that mediate the segregation of chromosomes to daughter cells during mitosis and meiosis. Because the centromeres of homologous chromosomes segregate from each other during the first meiotic division, loci near their centromere will tend to segregate in meiosis I (first division segregation), whereas crossovers between the centromere and more distal markers will lead to segregation of markers in meiosis II (second division segregation). The proportion of second division segregation at a locus is a function of the frequency of recombination between the locus and its centromere. Analysis of ordered tetrads—the four haploid products of a single meiotic division—can reveal how often a locus segregates at the first or second meiotic division, and hence the distance between the gene and its centromere (PERKINS 1949, 1953). Because recombination events are generally reciprocal, half-tetrads—one of the two products of the first meiotic division—give much the same information as an ordered tetrad (STREISINGER *et al.* 1986).

In mice and humans, a rare error in the meiotic arrest that follows meiosis I during oogenesis can result in ovarian teratomas derived from a single meiotic half-tetrad. Genetic analysis of these half-tetrads has led to the mapping of the centromeres of three mouse chromosomes (EPPIG and EICHER 1983; ARTZT *et al.* 1987; CHAKRAVARTI *et al.* 1989). [Other mouse centromeres have recently been localized by *in situ* hybridization for a strain specific centromere-associated satellite DNA (CECI *et al.* 1994).] In several species of fish, half-tetrads can be grown into adult diploid individuals routinely in the laboratory (ALLENDORF *et al.* 1986; STREISINGER *et al.* 1986). Analysis of such animals has shown that the

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pigment pattern mutation *rose* is tightly linked to the centromere of LG I (JOHNSON *et al.* 1995a), the CA-repeat variant *ssr12* to the centromere of LG XVII (KAUFFMAN *et al.* 1995), and the embryonic lethal mutation *no tail* to the centromere of LG XIX (HALPERN *et al.* 1993).

Because half-tetrad zebrafish express mutant phenotypes appropriate to their genotypes, we have proposed an efficient method for the initial mapping of mutations using centromere-linkage analysis (JOHNSON *et al.* 1995a). In a family of half-tetrad zebrafish, some will be homozygous for the mutant locus; in those homozygotes, most centromeres will segregate randomly, indicating that the mutation is unlinked to these centromeres. In contrast, for the centromere linked to the mutation, most or all of the selected homozygous mutant half-tetrads will have inherited the centromere allele in coupling to the mutant locus. Although we have used variations on this strategy to map several mutations, including the pigment pattern mutations *rose* (to LG I) and *jaguar* (to LG 15; JOHNSON *et al.* 1995a), efficient application of this technique will require the identification of markers tightly linked to the centromeres for each of the linkage groups of the zebrafish genetic map.

In work presented here, we report the identification of DNA polymorphisms closely linked to each of the 25 centromeres. This analysis plus the addition of 235 new markers to the map resulted in the consolidation of the number of linkage groups to the number of chromosomes. The application of centromere-linkage analysis to the large collection of unmapped zebrafish mutations promises to facilitate their genetic localization, and hence, the molecular genetic analysis of vertebrate development.

MATERIALS AND METHODS

Stocks: Mapping strains have been described previously (STREISINGER *et al.* 1981; JOHNSON *et al.* 1994, 1995a). Briefly, C32 (STREISINGER *et al.* 1981) is a clonal, presumably homozygous, derivative of STREISINGER's outbred population AB. The DAR (Darjeeling; JOHNSON *et al.* 1994) strain was isolated from fish captured in the wild in India in 1987, and subsequently brought to Eugene. The outbred DAR strain has not been maintained, and has been replaced by an inbred and isocentromeric isolate, SJD (JOHNSON *et al.* 1995a). The C32 and SJD strains are available on request.

Crosses and mapping panels: Half-tetrad and haploid mapping panels used have been described previously (JOHNSON *et al.* 1995a). Briefly, the half-tetrad mapping panel was generated by subjecting a C32 × DAR hybrid female to EP-parthenogenetic reproduction. A second half-tetrad mapping panel, generated from a C32 × SJD female, was used to provide additional mapping resolution for some centromeres (see Table 1). Half-tetrad embryos produced were allowed to develop for 3 days, then killed for DNA extraction (JOHNSON *et al.* 1994). The haploid mapping panel was generated from haploid progeny of a C32 × SJD hybrid female; haploids developed for three days, and were then sacrificed for DNA extraction. Segregation data for markers on these mapping panels is available on request.

Markers and nomenclature: Formal locus names for RAPD (WILLIAMS *et al.* 1990) markers in zebrafish consist of the name of the 10 nucleotide long primer, followed by the approximate size of the amplification product. Thus, the locus *10G.950*, on LG 20, is amplified by primer G10 (Operon Technologies; Alameda, CA), and results in a 950-bp amplification product. The formal locus name is followed by a letter in parentheses to indicate the parental origin of markers (A, AB; C, C32; D, DAR; S, SJD). Following this scheme, *10G.950* was originally designated as *10G.950(A)*, indicating that the AB-derived allele produced the amplification product. A slash separating two letters indicates that the marker is codominant in the two genetic backgrounds shown (*i.e.*, the locus produces slightly different sized PCR products from the different alleles, and haploid individuals in the mapping panel have one or the other sized product, but never both and never neither). To help present the data in Table 1, we have distinguished between the two alleles of codominant loci by indicating the size of their amplification products in the marker name. When a marker has been genotyped in both AB × DAR and C32 × SJD mapping panels, only the parental origin for the latter mapping panel, based on inbred strains C32 and SJD, is shown on the map. In some cases, the RAPD marker has been converted to a sequence tagged site (STS, see below, and Table 2). In these cases, the formal RAPD locus name remains the same, but is appended by a *, indicating that the marker may usually be detected in most genetic backgrounds, and the parental origin of the marker is omitted (for instance, *10G.950**).

Because consolidation of linkage groups has led to changing of some linkage group designations, and because the number of linkage groups, each with a mapped centromere, equals the number of chromosomes, we have renamed the linkage groups with Arabic, rather than Roman, numerals. Thus, formerly named LGs I–XVII, XX, XXII, XXIV and XXV (POSTLETHWAIT *et al.* 1994) have been renamed LG 1–17, 20,22, 24, and 25. In this study, former LGs XVIII and XXVIII were combined and renamed LG 18, LGs XIX and XXVI were combined and renamed LG 19, LGs XXI and XXIX were combined and renamed LG 21, and LGs XXIII and XXVII were combined and renamed LG 23.

Mapping: Methods for PCR amplification of RAPDs, simple sequence repeat (SSR) polymorphisms, and sequence tagged sites (STSs), as well the genotyping and the construction of the map have been described previously (POSTLETHWAIT *et al.* 1994). RAPD primers were chosen for genotyping this mapping panel based on their amplification of markers well distributed on the previous mapping panel (POSTLETHWAIT *et al.* 1994), and included primers that amplified markers on LG XXVI to XXIX or markers linked to *spa*, *ros* or *leo*, to integrate the two maps. To integrate the map constructed from the inbred C32 × SJD mapping panel with the previously published map constructed from the AB × DAR mapping panel, we averaged distances between markers common to both maps. Markers specific to one or the other map were then placed on the integrated map by proportioning the genetic distances accordingly.

The 95% confidence interval around centromere markers for probable centromere location was calculated according to the formula $Y/N \pm 1.96\,\{[(Y/N)(1 - Y/N)]/N\}^{1/2}$, where Y represents the number of heterozygous half tetrads for the indicated locus and N is twice the number of half-tetrads (because each half-tetrad represents two chromatids) (CROW 1950). If a locus is never heterozygous in the sample of half-tetrad individuals, Y in the second term was set equal to 1. For example, if a codominant centromere marker was not heterozygous in 20 half-tetrads, or a pair of dominant markers in repulsion segregated exclusively from each other, then the 95% confidence interval for centromere location extends 4.8

TABLE 1

Informative markers in centromere localization

Linkage group	Centromere marker or markers (fraction first division segregation)	Markers showing second division segregation	
		Limiting possible upper extent of centromere position	Limiting possible lower extent of centromere position
1	17X.900, 14AD.1600[a] (42/42)	18AF.550 (13Y.950/1000)[b]	2F.650
2	5N.1100* (20/20)	ND	7A.775
3	4C.890/900 (20/20)	ND	13B.1125
4	1B.850* (20/20)	ND	ND
5	1AD.1000, 7AE.540 (18/19)	ND (18A.820)	1AD.1000
6	6U.850, 14P.1350 (18/19)	5E.810* (6U.550)	ND (14P.1350)
7	5W.500/590 (20/20)	6G.380	ND
8	6O.540/560 (20/20)	DAB	7N.900
9	1L.1020/1040 (19/20)	2N.430	16J.1270 (8A.375)
10	18AF.430/440 (20/20)	13Y.1550	ND
11	8B.875/1000 (20/20)	4W.700* (8B.875/1000)	ND
12	14U.800, 4M.1550/1600 (18/18)	12M.620	18A.490
13	7A.1450* (20/20)	ND	7A.800/810
14	15O.810/820 (19/19)	10AF.1120	ND (6U.1450)
15	2AI.600, 11AA.1500 (18/18)	14P.820	11AA.1500/1600
16	18A.475, 13Y.1700 (20/20)	14AD.700 (15N.675)	ND
17	ssr12 (20/20)	ND (18A.830)	17X.675
18	6AB.410/425 (20/20)	9AB.1290	6AC.870
19	no tail (20/20)	ND	13Y.1600
20	15AA.625, 14U.1300 (18/18)	ND	10G.950*
21	5H.420/430 (20/20)	14P.1050*	6U.425
22	4C.1330, 2AD.650 (18/20)	4A.1040	2AD.650 (2AA.930)
23	13C.800/810 (20/20)	1I.900 (4X.525)	18A.1275 (O15.375)
24	5O.950, 20V.1600 (15/19)	20V.1600	5O.950
25	6AC.525, 16AI.590 (18/19)	20B.550/560	6AC.525

[a] From JOHNSON *et al.* 1995a.

[b] In parentheses, markers exhibiting second division segregation in a second C32 × SJD half-tetrad panel that allowed for additional limitation of possible centromere location.

cM on either side of the codominant centromere marker or pair of dominant markers in repulsion. In some instances, second-division segregation of markers located within the 95% confidence interval allowed further restriction of the centromere location (see Table 1 and Figure 2).

Conversion of RAPD markers to STSs: To help in relating maps from different haploid and half-tetrad mapping panels, we cloned some RAPD markers, sequenced the ends of the clones, and generated primer pairs that specifically amplify the original RAPD locus as confirmed by segregation analysis. STS markers derived from RAPD markers are either size polymorphic, amplify product from one or the other genetic background only, or are polymorphic at restriction enzyme recognition sites. These STSs should be generally useful in mapping crosses involving many different strains. A list of STSs used in centromere mapping and construction and integration of maps is provided in Table 2.

RESULTS

Identifying DNA polymorphisms tightly linked to each centromere: The first step in localizing centromeres on the zebrafish linkage map was to identify genetic markers closely linked to each centromere. If a marker is located near the centromere (for example, locus *C* in Figure 1A), then a cross-over will rarely occur between the locus and the centromere. Consequently, most or all half-tetrads will be homozygous for one or the other of the two alleles at this locus. In contrast, if a marker is located far from its centromere (for example, locus *A* in Figure 1A), a cross-over will often occur somewhere between the locus and the centromere. The result of this cross-over is a half-tetrad that is heterozygous for markers distal to the point of the cross-over.

Loci not located near the centromere (for example, loci *A* and *B* in Figure 1A), can be ordered relative to each other and the centromere using data collected from half-tetrads because a single cross-over between the two loci results in animals homozygous for the locus nearer the centromere, but heterozygous for the locus further from the centromere. A cross-over on one side of the centromere should not affect the genotype of a locus on the opposite chromosome arm (for example, loci *A* and *D* in Figure 1A).

To identify loci near each centromere, half-tetrad progeny from a C32 × DAR hybrid female were genotyped for markers distributed along each linkage group. An example of the analysis is shown in Figure 1B. This figure illustrates the genotype for the two chromatids of each of 20 half-tetrad individuals. Consider first the locus *6O.540,* a codominant locus at which the RAPD primer O6 (Operon Technologies, Alameda, CA) amplifies a 540-bp band from the C32-derived allele and

TABLE 2

STS markers near centromeres and others used in making map

Linkage group and marker	Primer pair sequences	Product size	Basis for polymorphism
LG 2 5N.1000*	CGCCTGACTGATTCTCAAAG GAACGCCACAAAACCAATAG	950	*Hin*FI, *Nla*III
LG 3 15R.600*	GACAACGAGGACACGAACTG AACGAGATGCTGCGCTGCC	575/600	Size
8A.500*	TGACGTAGGCTGGTAATTGG TGACGTAGGAGTGCAGTTTG	490/500	Size
LG 4 1B.850*	CGCTCCTAACTGGTTTTCCA TCCACCTTCAGGATACTGGC	850	*Dpn*II
LG 5 2AD.925*	GCTCTGACCAACATACTTCACG CAGACTCATCAGACCAGGCA	800	SJD only
LG 6 5E.810*	GGAGGTCACAGAGTTATGCTC TCAGGGAGGTGTGGAAATGC	800/810	Size
LG 10 4Y.350*	GGAGCTGCTTCTTCAAAACG TGGTAGAACCTGTCTGTATGG	260/280	Size
LG 11 4W.700*	GAGAAGCGGACCTAAAATGC CAGAAGCGGAAGGCGCGAG	650/700	Size
LG 13 7A.1450*	GAAACGGGTGTATATAATAAA GATTTTGTACAGAGATTATAA	1300	*Mse*I
7A.800*	GAAACGGGTGGTCGACTACAA GAAACGGGTGCCTGTAGTGTA	790/810	Size
LG 19 no tail	CCTCCTCAATGTACGATCCA TCAAAGAGCCCAACAAATACA	920	*Hin*FI
LG 20 10G.950*	CCGTCTTGAGGCGTTTTACC AGTCCTCCATTGTGGTGGGC	950	*Hae*III, *Nla*III
LG 21 14P.1050*	GGACAGACGCAAGTATCCAC GCACTCTCCACCGCATTTCC	820	*Alu*I
msxD	CGGAACATTTTAGCTCCGTC GTGGTGGACATTAACTCATCCA	210/230	Size
LG 22 2AD.650*	GGGAAACACCCATACACACC GGTGCTGTGAGATCAAAAGATG	520	*Msp*I
LG 24 13B.1600*	CCACGTTCATATTGCCTGTG CCCGCTCTGCATATTGTGTA	1400/1500	Size

a 560-bp band from the DAR-derived allele. Linkage analysis had shown that locus *6O.540* lies on LG VIII (Figure 2). About half (11/20) of the half-tetrads in the panel are homozygous for the C32-derived allele of *6O.540* and about half (9/20) are homozygous for the DAR-derived allele. Because none of these half-tetrads (0/20) are heterozygous, we conclude that *6O.540* maps near the centromere of LG VIII.

In contrast to *6O.540,* the codominant *MHC* class II locus *DAB,* located ~4 cM from *6O.540,* (Figure 2), shows one heterozygous half-tetrad (half-tetrad number 15, Figure 1B) in the panel of 20 half-tetrad individuals shown. This heterozygous half-tetrad resulted from a crossover between *DAB* and its centromere. Because the *6O.540* locus is homozygous in this half-tetrad, we can conclude that the crossover occurred between *6O.540* and *DAB.* This result shows that the centromere is not in the region of LG VIII above *DAB,* as oriented in Figure 1B.

While codominant loci like *6O.540* and *DAB* are especially useful for analyzing half-tetrads, simple dominant loci can also provide valuable information. As an example, consider *7N.900,* a simple dominant locus that produces a 900-bp amplification product from DAR-derived allele but no detectable amplification product from the C32-derived allele. Because a homozygote carrying two DAR-derived alleles of locus *7N.900,* and a heterozygote carrying one DAR allele and one C32 allele of this locus have the same phenotype—a 900-bp amplification product from primer N7—the DAR-derived allele is dominant to the C32-derived allele of *7N.900.* If there were no crossovers between *6O.540* and *7N.900,* then all half-tetrads homozygous for the codominant C32 allele of *6O.540* should also be homozygous for the recessive C32-derived allele of *7N.900,* and hence lack the *7N.900* band. Of the 11 half-tetrads in the panel that are homozygous for the C32-derived allele of *6O.540,* four individuals (numbers 4, 6, 7, and 14) amplify the 900-bp product from primer 7N, and thus have at least one copy of the DAR-derived allele of locus *7N.900* (Figure 1B). These four half-tetrad individuals thus derive from meioses in which a cross-over occurred between *6O.540* and *7N.900.* These results show that the centromere of LG VIII is not below the *7N.900* locus, as shown in Figure 1B. Together, the analysis of the three loci *DAB, 6O.540,* and *7N.900* indi-

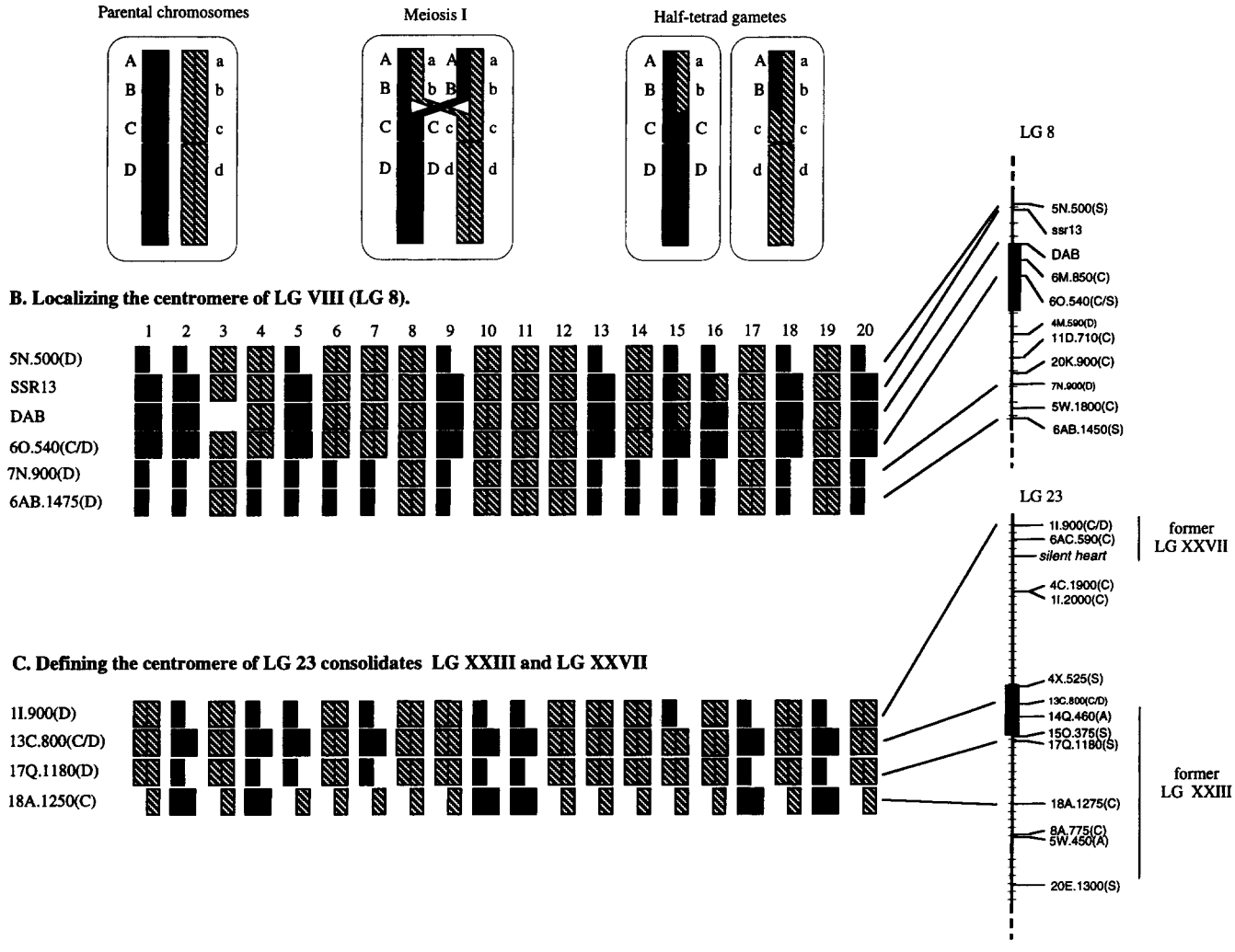

FIGURE 1.—Centromere-linkage analysis. (A) Segregation of markers in half-tetrads. A female from a hybrid mapping cross is heterozygous for markers *A, B, C,* and *D* along the length of her chromosomes (left). Crossing-over between markers *B* and *C,* which resides near the centromere (middle), followed by disruption of the second meiotic division, results in half-tetrad cells that are homozygous for markers proximal to the crossover (centromeric marker *C*), and heterozygous for markers distal to the crossover (markers *A* and *B*). (B) Localizing the centromere of LG VIII. The genotypes of markers on LG VIII were determined for 20 half-tetrad progeny of a C32 × DAR female. The loci listed at the left are arranged according to their order on the genetic map, and the genotype of each half-tetrad is depicted as its two chromatids to help visualize heterozygotes and the location of crossovers. Dark shading indicates inheritance of the DAR-derived allele; cross hatching indicates inheritance of the C32-derived allele (See MATERIALS AND METHODS for further discussion about marker nomenclature). If a half-tetrad is homozygous for a codominant or recessive marker, then both chromatids are shaded the same. If a half-tetrad is heterozygous for a codominant locus, then each chromatid is shaded differently. If a half-tetrad shows the phenotype of a dominant locus, then the genotype of one chromatid is unknown, and so one side of the box is left blank. For example, half-tetrad 4 is homozygous for the C32-derived alleles of all loci between *5N.500* and *6O.540,* but a cross-over between *6O.540* and *7N.900* lead to a portion of the half-tetrad that bears at least one copy of the dominant alleles of *7N.900* and *6AB.1475.* In the case of missing data, no shaded chromatids are depicted. The central portion of LG VIII is shown at the right of the figure, with the inferred 95% confidence interval for the centromere position depicted as a black rectangle (each cross-hatch on the map represents 2 cM). (C) Defining the centromere of linkage group 23 consolidates LGs XXIII and XXVII. Codominant RAPD marker *13C.800* is homozygous in all members of the panel, and hence must lie near the centromere. Only one recombinant (individual 15) is apparent between the LG XXIII marker *11.900* and the centromere-proximate marker *13C.800,* showing that the two markers are linked. Similarly, no recombinants occur between the LG XXVII marker *17Q.1180* and *13C.800,* and only two recombinants (individuals 5 and 7) are apparent between *18A.1350* and *13C.800.* Markers for the other 24 centromeres do not cosegregate with markers from LG XXIII and LG XXVII (for example, compare segregation of these homozygous half-tetrads with segregation of the centromere of LG VIII, depicted in Figure 1B). Marker order for the consolidated linkage group was indicated by analysis of the haploid mapping panels. Because *13C.800* failed to segregate in the haploid mapping panels, its position on the LG 23 map is inferred from centromere segregation analysis of a second C32 × SJD half-tetrad panel, that placed the centromere between markers *4X.525* and *15O.375.*

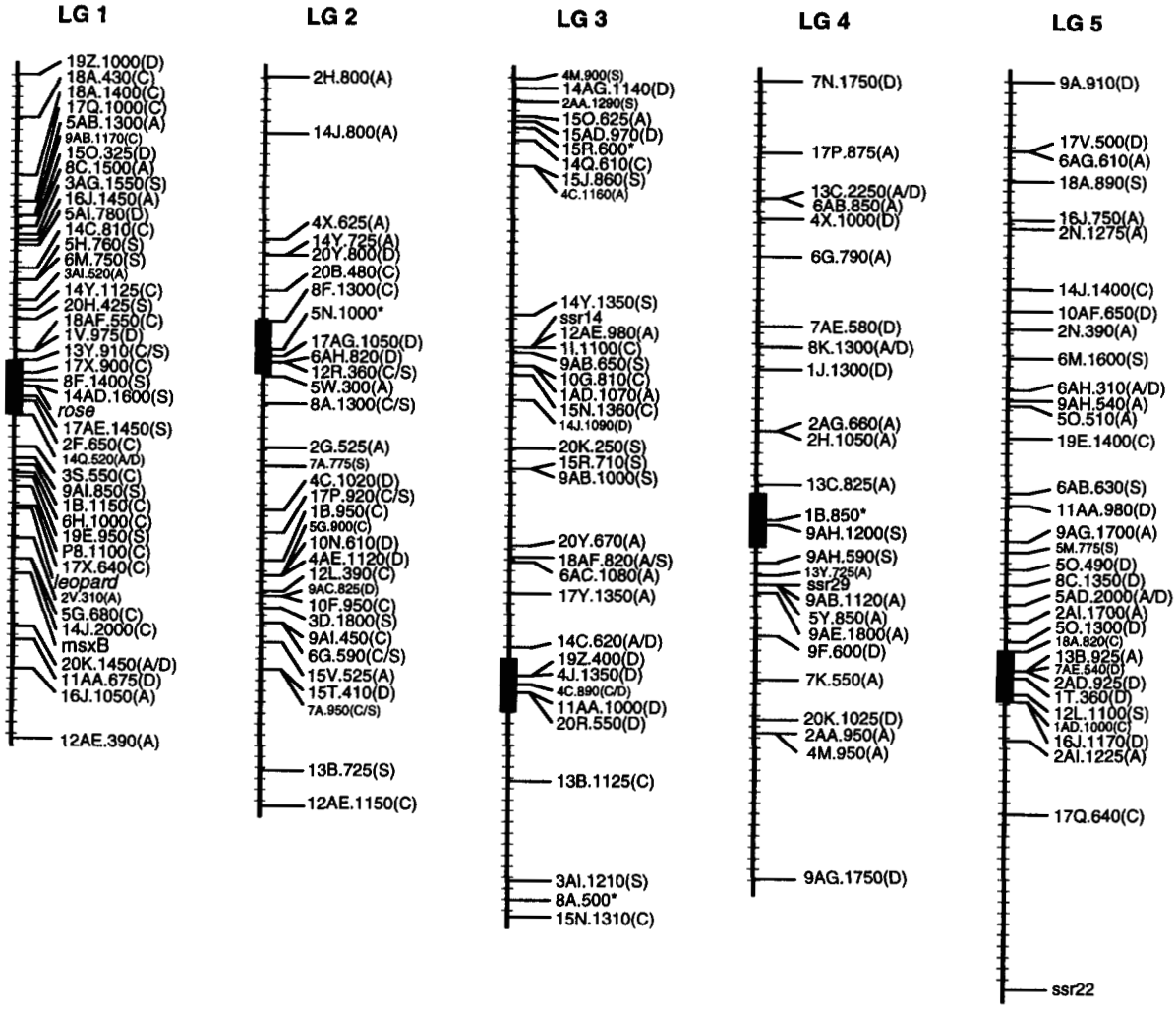

FIGURE 2.—A consolidated linkage map for the zebrafish. This linkage map shows the positions of 652 markers genotyped in one or both of two haploid mapping panels. Centromere locations determined by half-tetrad analysis are shown as black rectangles (95% confidence interval). RAPD marker names indicate the primer name assigned by Operon Technologies, Inc., followed by the approximate size in base pairs of the amplified marker. Letters in parentheses indicate the parental origin of markers (A, AB; C, C32; D, DAR; S, SJD). Parentheses containing two letters separated by a slash (C/S) indicate a codominant marker. See MATERIALS AND METHODS for further discussion of nomenclature. Markers shown in a smaller font have some uncertainty of local marker order due to integration of maps from the two mapping panels. RAPD markers converted to STSs are indicated by their RAPD name followed by a * (star, Sequence Tagged RAPD). Cross bars are shown every 2 cM.

cate that the centromere must lie between *DAB* and *7N.900* in the vicinity of *6O.540*.

Analysis similar to that described in Figure 1B for LG VIII served to localize 24 of the 25 centromeres—those residing on LGs I (JOHNSON *et al.* 1995a), II to XXII, XXIV, and XXV. Markers used in the half-tetrad mapping panels to limit the location of the centromeres are listed in Table 1. These experiments confirmed the previously identified centromere locations for LGs XVII and XIX (HALPERN *et al.* 1993; KAUFMANN *et al.* 1995), and left one centromere yet to be defined. The codominant RAPD marker *13C.800* showed no heterozygotes in all 20 members of the C32 × DAR half-tetrad panel, but segregated independently of the other 24 centromeres, suggesting that *13C.800* identifies the last centromere. Because *13C.800* failed to segregate in either haploid mapping panels (see below), further analysis was needed to determine to which of the published 29 linkage groups *13C.800* belongs.

The net results of these experiments was the identification of DNA polymorphisms closely linked to each of the 25 centromeres in zebrafish.

Centromere-linkage analysis and the consolidation of linkage groups: The identification of markers near each of the 25 zebrafish centromeres provided genetic resources for consolidating the number of linkage groups to the number of chromosomes using centromere-linkage analysis. For centromere-linkage analysis (JOHNSON *et al.* 1995a), one first collects a family of half-tetrads segregating DNA polymorphisms and the marker or mutation to be mapped. Next, one identifies those half-tetrads that are homozygous for the desired mutation or marker and determines their genotypes for markers closely linked to each of the 25 centromeres.

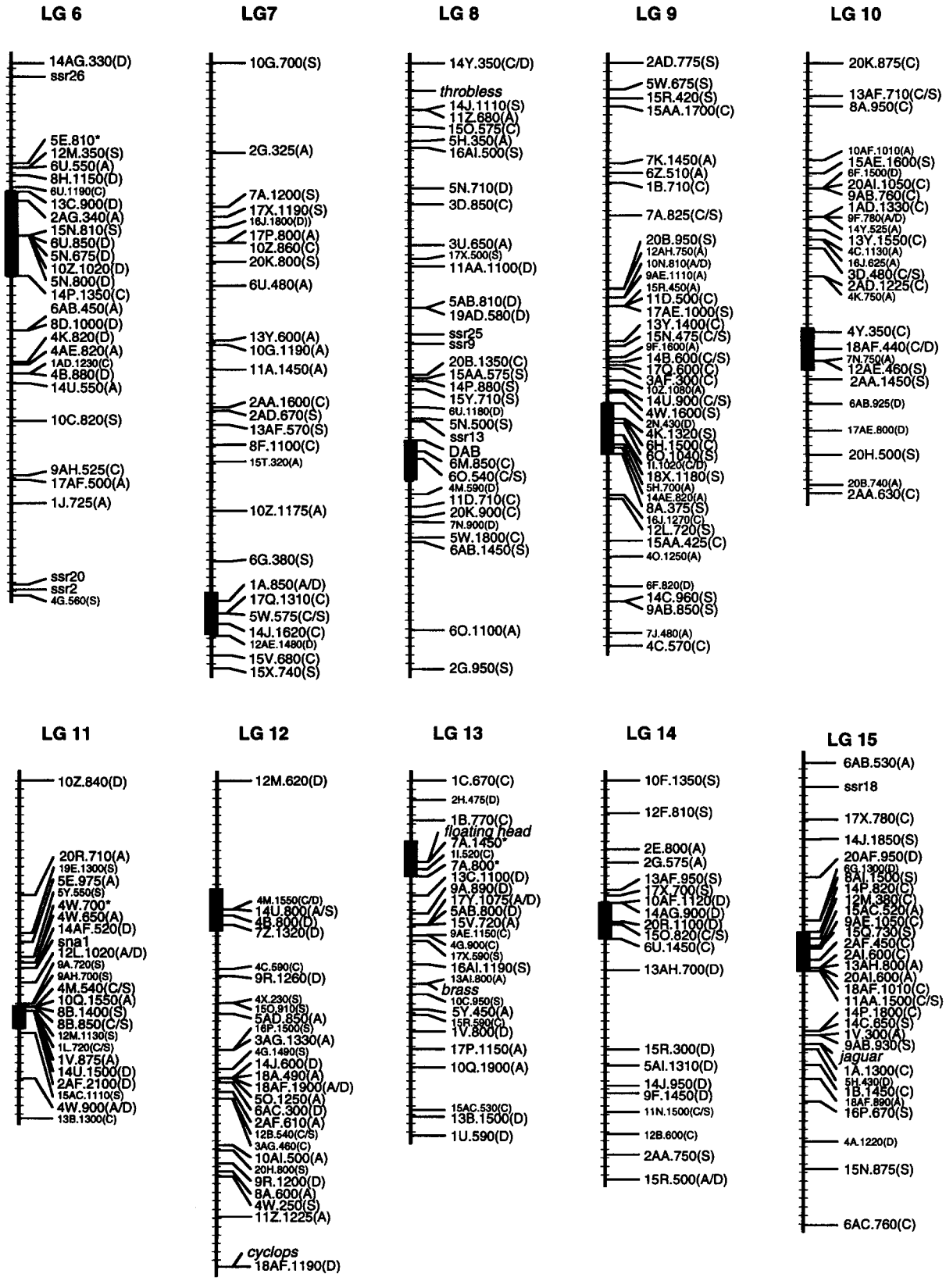

FIGURE 2.—*Continued*

Half-tetrads in this subset will almost always be homozygous for the allele of the centromere marker to which they are linked in coupling (*i.e.*, in *cis*). The only exceptions would arise in infrequent cases when the centromere-linked marker was itself heterozygous or from rare four-strand double cross-overs between the mutant locus and its centromere marker. In the latter case, half-tetrads homozygous for the marker to be mapped

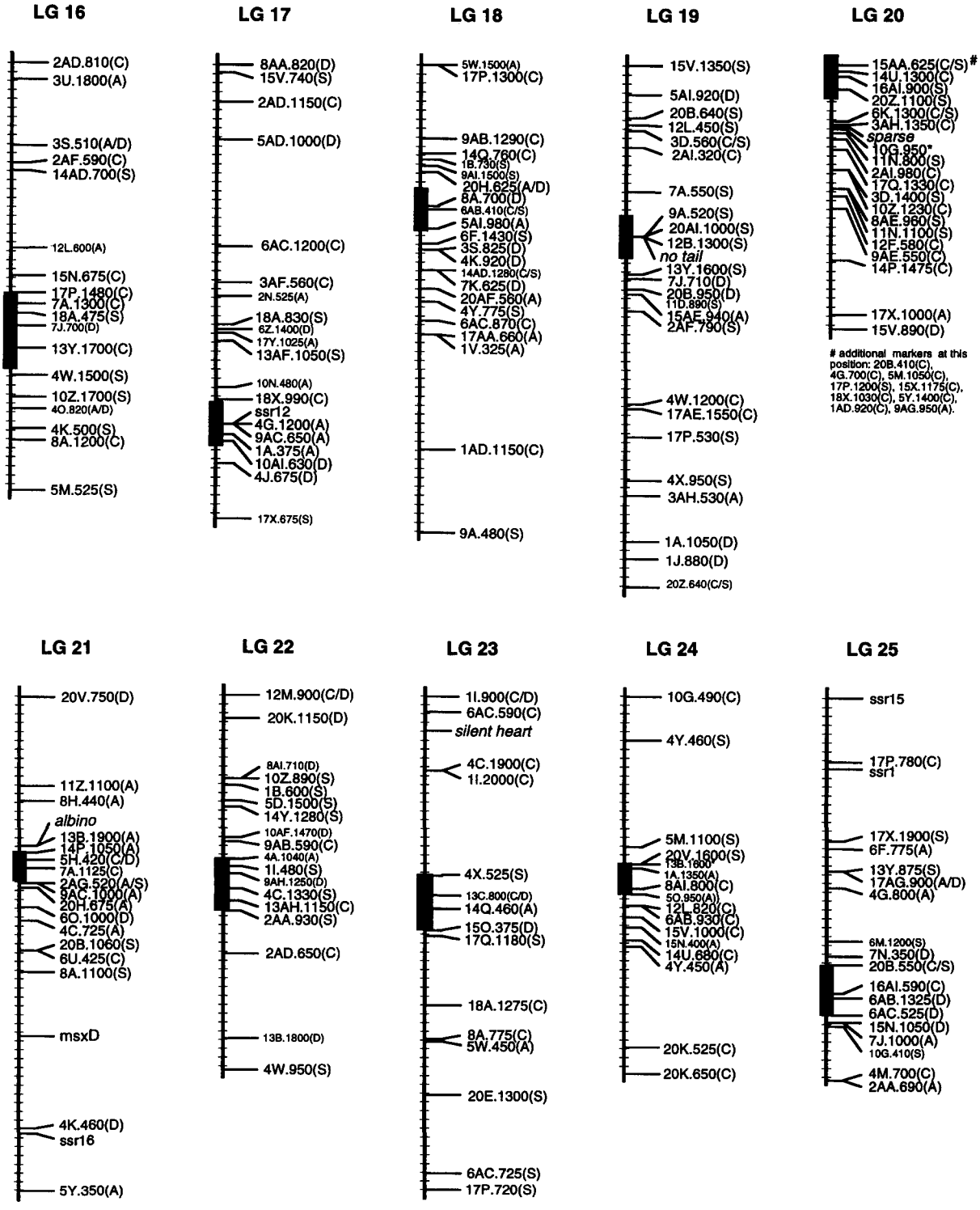

FIGURE 2.—*Continued*

would also be homozygous for the allele of the centromere marker linked in repulsion (*i.e.*, in *trans*). In contrast, centromere markers unlinked to the mutation or marker of interest will segregate randomly in the selected set of homozygous half-tetrads.

We used half-tetrad centromere-linkage analysis to identify which of the 25 centromeres are linked to LGs XXIII, XXVII and XXIX, linkage groups for which centromere locations had not been established in the foregoing analysis. As an example of this centromere-linkage analysis, consider the experiment that mapped LG XXIII to its centromere (Figure 1C). The dominant RAPD marker *17Q.1180* was previously mapped to LG XXIII (POSTLETHWAIT *et al.* 1994). Because primer Q17 amplifies an 1180-bp product from the DAR-derived chromosome, but not from the C32-derived chromosome, the subset of half-tetrads that failed to amplify the *17Q.1180* band were homozygous for the recessive C32-derived al-

lele; thus, the 11 half-tetrads 1, 3, 6, 8, 9, 12, 13, 14, 15, 18, and 20 in Figure 1C are identified as homozygotes for the locus to be mapped. To test whether *17Q.1180* is linked to the centromere of LG VIII, we determined whether the 11 selected half-tetrads were also homozygous for the C32-derived allele of LG VIII's centromere marker *6O.540*. Figure 1B shows that five (3, 6, 8, 12, 14) of the 11 half-tetrads homozygous for the marker to be mapped are homozygous for the C32-derived allele of the LG VIII centromere marker, while six (1, 9, 13, 15, 18, 20) are homozygous for the DAR-derived allele. This independent segregation shows that *17Q.1180*, and hence LG XXIII, is not linked to the centromere of LG VIII. Similar results tended to exclude the possibility of linkage of LG XXIII to the centromeres of LGs I-XXII, XXIV and XXV.

Contrasting results were obtained when the LGXXIII marker *17Q.1180* was checked for linkage to the centromere marker *13C.800* (Figure 1C). All 11 members of the subset of half-tetrads homozygous for the recessive C32-derived allele of *17Q.1180* (1, 3, 6, 8, 9, 12, 13, 14, 15, 18, and 20) were also homozygous for the C32-derived allele of the centromere marker *13C.800*. This shows that *13C.800* marks the centromere of LG XXIII.

Centromere-linkage analysis similar to that just described was conducted for the LG XXVII marker *1I.900* (Figure 1C). Results showed that the subset of half-tetrads that are homozygous for the recessive C32-derived allele of *1I.900* (1, 3, 6, 8, 9, 12, 13, 14, 16, 18, and 20) are also all homozygous for the C32-derived allele of the same centromere marker to which LG XXIII is linked, *13C.800* (Figure 1C). These data show that LG XXIII and LG XXVII are both linked to the same centromere and hence must be linked to each other. The new consolidated linkage group is called LG 23.

Similar analysis showed that the subset of six half-tetrads in the panel of 20 half-tetrad embryos that are homozygous for the recessive DAR-derived allele of the new marker *6U.425* on LG XXIX are also all homozygous for the DAR-derived allele of 5H.420, which marks the centromere of LG XXI. This indicates that former linkage groups XXI and XXIX are linked to the same centromere and hence make consolidated LG 21. Markers for the two small linkage groups LG XXVI and LG XXVIII did not appear to segregate in our family of half-tetrads, so they could not be mapped by centromere-linkage analysis.

As a result of these experiments, the number of linkage groups was reduced from the 29 previously that were published (POSTLETHWAIT *et al.* 1994) to 27, still two more than the number of chromosomes.

Adding new markers to the map and the consolidation of linkage groups: To close the remaining gaps in the map, we established a new haploid mapping panel and genotyped it for additional markers. We reasoned that addition of new markers to the map might identify some that fall in gaps on the map and thereby establish linkage between distantly linked markers or apparently independent linkage groups. The new haploid mapping panel was based on 96 haploid progeny from a hybrid female offspring from the mating of the clonally derived strain C32 and the inbred strain SJD. We genotyped these haploids for many of the markers on the previous map, including markers near the ends of most of the 29 linkage groups described previously as well as markers generated by primers not used in construction of the first map (POSTLETHWAIT *et al.* 1994).

The map generated from the new haploid mapping panel was nearly as complete as the previously published map (POSTLETHWAIT *et al.* 1994). A total of 374 markers were genotyped on the new panel, including 355 RAPD markers, 14 SSRs and five STSs located within the 3′ untranslated regions of the genes *mhc DAB* (ONO *et al.* 1992), *msxB* (AKIMENKO *et al.* 1995), *snail1* (THISSE *et al.* 1993), *no tail* (HALPERN *et al.* 1993), and *msxD* (EKKER *et al.* 1992). The 355 RAPD markers included seven that failed to show linkage to any other markers. Some markers (132) genotyped in the C32 × SJD mapping panel had been previously placed on the map generated by the AB × DAR mapping panel (POSTLETHWAIT *et al.* 1994), facilitating integration of the two data sets. Analysis of the segregation data for the C32 × SJD mapping panel with MapMaker (LANDER *et al.* 1987) revealed 33 linkage groups, suggesting that this second map may have as few as eight gaps. Common markers aligned the original AB × DAR map with the new C32 × SJD map, generating a composite map. As hoped, markers in the second map complemented the gaps in the first, resulting in a composite map with 652 PCR-based markers, 11 mutant loci and 25 linkage groups (Figure 2).

Some of the 235 newly localized markers fell in each of the four gaps in the earlier map. For example, *4C.1900, 1I.2000,* and *4X.525* reside in the gap between LGs XXIII and XXVII in the order shown in Figure 2. This result confirmed the consolidation of LGs XXIII and XXVII that was established by centromere-linkage analysis, and ordered the two former linkage groups with respect to each other and their shared centromere in new LG 23. Similarly, the localization of new markers *msxD, 8A.1100, 6U.425,* and *20B.1060* confirmed the linkage between former LGs XXI and XXIX that was established by centromere-linkage analysis and provided information for marker order and recombination distances for a consolidated LG 21 (Figure 2). Additionally, LGs XIX and XXVI were consolidated into new LG 19 by bridging the gap with new markers *4W.1200, 17AE.1550,* and *17P.530*. LGs XVIII and XXVIII were consolidated to LG 18 by the finding that in the C32 × SJD mapping panel, markers *6AC.870* (LGXVIII) and *1AD.1150* (LG XXVIII) showed significant linkage (13 recombinants in 69 embryos genotyped for both markers; LOD = 12.1).

The net result from the half-tetrad analysis and the addition of new markers to the genetic map was the consolidation of the genetic map of zebrafish to 25

linkage groups, the same as the number of centromeres and chromosomes.

DISCUSSION

The experiments presented here identified DNA polymorphisms closely linked to each of the 25 centromeres in the zebrafish genome, placed these loci—and hence centromeres—on the genetic linkage map, brought the total markers on the zebrafish map to 652, thereby giving an average marker density of one marker per 4.3 cM and consolidated the number of linkage groups to the number of chromosomes. The zebrafish is the fourth vertebrate, after the human, mouse, and rat, and the first nonmammalian vertebrate, with the same number of linkage groups as chromosomes and centromeres placed on the linkage map.

Centromere-linkage analysis: The identification of DNA polymorphisms closely linked to each of the 25 zebrafish centromeres has practical value for mapping the hundreds of unmapped mutations recently identified in zebrafish (KIMMEL 1989; MULLINS and NÜSSLEIN-VOLHARD 1993; DRIEVER *et al.* 1994; HENION *et al.* 1995; JOHNSON and WESTON 1995; JOHNSON *et al.* 1995b), because centromere markers facilitate the use of half-tetrad centromere-linkage analysis. Centromere-linkage analysis for mutations includes three steps. First, the fraction of homozygous mutants in a group of half-tetrads helps define the distance between the mutation and its centromere (STREISINGER *et al.* 1986). Second, identifying the subset of half-tetrads homozygous for the mutation and genotyping them with the centromere markers identified here locates the mutation to a linkage group (JOHNSON *et al.* 1995a). Assessing as few as six, but ideally 10–12 homozygous mutant half-tetrads for segregation of each of the 25 centromeres should identify to which linkage group the mutation belongs, because its centromere will segregate nonrandomly with the mutant locus. Third, the location of the mutation on the linkage group can be determined by analysis of other markers on the linkage group in half-tetrads. Loci on the opposite chromosome arm, or distal to the mutation on the same chromosome arm, will sometimes be heterozygous in the mutant half-tetrads, whereas markers between the homozygous mutant locus and its centromere will usually be homozygous (with the exceptions of infrequent double crossover half-tetrads). At this stage, increased mapping resolution may be easily achieved by increasing the number of homozygous mutant half-tetrads analyzed. These initial phases of linkage analysis may require only ~300 PCR assays.

Centromere-linkage analysis is an effective first step in linkage studies because it allows the investigator to concentrate analysis on a subset of chromosomes that are nonrecombinant, or have few crossovers, between the mutant locus or marker of interest and its centromere. For instance, in an appropriate mapping cross, half-tetrads homozygous for the *leopard* mutation, a locus ~26 cM from its centromere, showed a 32:0 segregation ratio for the centromere allele in coupling *vs.* the centromere allele in repulsion to the *leopard* mutation (JOHNSON *et al.* 1995a). Because multiple crossovers do occur on zebrafish chromosomes (JOHNSON *et al.* 1995a), the absolute exclusion of the centromere allele in repulsion may not hold for larger intervals. Nevertheless, in the absence of interference, loci at distances of even 100 cM from their centromeres should show ratios of 2.2 to 1 of the centromere allele in coupling to the centromere allele in repulsion to the mutation (PERKINS 1953; JOHNSON *et al.* 1995a); such a ratio may still be useful for detecting linkage if enough animals are used. Because only a small fraction of the zebrafish map extends further than 100 cM from the centromere (the upper arms of LGs 3, 5, and 7 are ~111 cM, 110 cM, and 127 cM total length, according to the current map), application of centromere-linkage analysis should help localize mutations that lie on almost any part of the map.

Results presented here demonstrate the utility of centromere-linkage analysis for assigning mutations or genetic markers to linkage groups. Although centromere-linkage analysis is particularly convenient in zebrafish because half-tetrad individuals can be grown to adults and express mutant phenoytpes, it can in principle be used in any species by the PCR amplification of markers from individual primary oocytes after removal of the first polar body, as has been demonstrated for mouse centromere mapping studies (CUI *et al.* 1992).

The centromere markers used here are likely to be generally useful in any highly polymorphic mapping crosses that employ one or the other of the inbred mapping strains C32 or SJD. In addition, we have already established sequence tagged sites (STSs) closely linked to 13 of the 25 centromeres (Table 2), and are in the process of making STSs from RAPD markers closely linked to the remaining centromeres. Identifying a restriction enzyme polymorphism in the amplification products of each STS in the two parental backgrounds of any mapping cross will provide appropriate markers to perform centromere-linkage analysis. Once identified, these polymorphisms should be informative in crosses involving all mutations isolated on the same inbred background.

The consolidated linkage map: The consolidation of the zebrafish map initiated by centromere-linkage analysis was confirmed and augmented by the addition of 235 new markers to the map. The new map has 652 PCR-based markers, including 14 SSRs (GOFF *et al.* 1992), five STSs located within genes, 619 RAPD markers, and 14 STSs constructed from RAPDs (PARAN and MICHELMORE 1993). We are in the process of developing STSs from RAPD markers which are distributed evenly across the genome; these markers will be useful in mapping crosses that do not employ the inbred or clonal genetic backgrounds used in the construction of the current map.

Because the number of linkage groups is now equal

to the number of haploid chromosomes, because centromeres have been positioned on each linkage group, and because almost all informative markers (367/374 markers genotyped in the C32 × SJD mapping panel) are linked to other mapped loci, most of the zebrafish genome is now represented in the current map. Because telomeres have not yet been placed on the map, additional markers may yet be identified that are located beyond the boundaries of the current map but linked to the terminal markers shown. The consolidated map spans ~2790 cM with an average interval of ~4.3 cM between markers. Adding the estimated distance between the terminal markers and the telomeres [$^1/_2$ the average interval between loci for each telomere, or $^1/_2$ (4.3 × 50) = 108] to the mapped region gives an estimate for the size of the entire female genetic map of ~2900 cM. This is about the same as our previous minimal estimate of 2720 cM (POSTLETHWAIT *et al.* 1994). Because the haploid genome of zebrafish contains ~1.7 × 10^9 bp (HINEGARDNER and ROSEN 1972), there are ~590 kbp/cM, an important parameter when considering chromosome walking experiments.

The location of centromeres on the linkage map can be compared with their cytological location on physical chromosomes. All linkage groups described here have markers on two arms except LG 20, which has a cluster of markers near its apparently terminal centromere. In contrast, karyotypic analysis has shown that all zebrafish chromosomes are metacentric, submetacentric, or subtelocentric (ENDO and INGALLS 1968; DAGA *et al.* 1996). A hypothesis to explain this apparent contradiction is that the C32 and SJD strains may differ by a chromosome rearrangement, such as an inversion, on the upper arm of LG 20. Such chromosome aberrations may disrupt chromosome pairing during meiosis and interfere with recombination. As a consequence, genetic markers which reside in the region of the rearrangement might appear to cluster as a single point on the recombination map. Alternatively, we may have yet to identify markers on the upper arm of LG 20. Cytogenetic experiments using probes developed from markers in the centromere region of LG 20 may help to resolve this issue.

The consolidated map for the zebrafish (Figure 2) can be compared to the maps of other vertebrate genomes. Among mammals, the human (MURRAY *et al.* 1994), mouse (COPELAND *et al.* 1993; DIETRICH *et al.* 1994) and rat (YAMADA *et al.* 1994; JACOB *et al.* 1995) maps are the most complete, with >6000, 4000, and 500 loci identified in each, respectively, and the same number of linkage groups as chromosomes. Progress has also been extensive for maps in other mammals: the maps of swine (ARCHIBALD *et al.* 1995; JOHANSSON *et al.* 1995) cattle (BARENDSE *et al.* 1994), and sheep (BROAD and HILL 1994) contain ~250, 200, and 100 loci, and only a few more linkage groups than chromosomes. Among nonmammalian vertebrates, the chicken map is especially well developed (BURT *et al.* 1995), and the genetic maps of the swordtail *Xiphophorus* (MORIZOT 1994) and salmonids (MAY and JOHNSON 1993) are particularly rich in loci encoding various isozymes. The addition of the homologues of genes mapped in these other vertebrate species to the consolidated map of the zebrafish should contribute to understanding how the vertebrate genome evolved (MORIZOT 1994).

Finally, the genetic resources reported here make possible the rapid mapping of zebrafish mutations to unique locations, and should therefore facilitate the localization of the hundreds of unmapped zebrafish mutations. Comparing these locations with the map positions of cloned genes should help reduce the number of candidate genes for each mutation; in favorable cases, such as with *floating head* (TALBOT *et al.* 1995), this will lead to the molecular identification and isolation of the mutated gene. Alternatively, the construction of marker-dense genetic maps, such as the map presented here, will aid in chromosome walks to the mutated gene. The work reported here should thereby contribute to our understanding of the genetic mechanisms of developmental processes held in common by all vertebrates, including humans.

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