SEGREGATION ANALYSES AND GENE-CENTROMERE DISTANCES IN ZEBRAFISH

GEORGE STREISINGER,[1] FRED SINGER, CHARLINE WALKER,[2] DONNA KNAUBER AND NANCY DOWER

Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

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ABSTRACT

The *gol-1*, *gol-2*, *alb-1* and *spa-1* mutations affect pigment pattern in the zebrafish. We show here that these loci are unlinked to each other. In addition, gene-centromere distances were determined for these loci by analysis of half-tetrads obtained by the inhibition of the second meiotic division. The fractions of tetratype (second-division segregation) tetrads range from 0.24 (*spa-1*) to 0.89 (*gol-1*). The observation of >0.67 second-division segregation indicates that the zebrafish has high chiasma interference.

LINKAGE analysis is simple in organisms that develop from haploid spores, because the phenotypes of segregants of crosses reflect their genotypes. A similar situation can be arranged with zebrafish in two different ways (STREISINGER *et al.* 1981). First, eggs can be fertilized by inactive sperm to generate haploid zygotes that can be treated after fertilization to produce homozygous diploids. Second, diploid gametes can be produced by treatments that block the second meiotic division of the egg. Fertilization with inactive sperm then yields embryos whose phenotype reflects the egg genotype. Thus, linkage analysis in zebrafish can be accomplished without the need for back-crosses to double mutant strains or for crosses among F_1 individuals.

Here, we describe the independent segregation of mutations in genes that affect pigment patterns in zebrafish. Some of these mutations were used to measure γ-ray-induced specific site mutation frequencies (CHAKRABARTI *et al.* 1983). The studies reported here demonstrate that the genes are not closely linked and, hence, validate our previous supposition that they are independent loci.

Large-scale linkage analyses are cumbersome because a separate cross is required for each pair of mutants to be examined. A simpler procedure is to measure distances between the sites of mutations and their centromeres: If each of several mutations lies at a very different distance from a centromere, the mutations cannot be closely linked to each other. For the detection of close linkage, crosses need to be performed only between pairs of mutants which

[1] Deceased.
[2] To whom reprint requests should be addressed.

occupy similar positions relative to their centromeres. Here, we describe measurements of the distances of pigment-pattern genes from their centromeres. Gene-centromere intervals can be measured in meiotic half-tetrads, and, as shown by the ovarian teratoma method in the mouse (EPPIG and EICHER 1983), gene-centromere and gene-gene distances obtained this way are equivalent to those measured with classical backcross methods. To measure gene-centromere distances we produced meiotic half-tetrads by inhibiting the second meiotic division, as has been done for other lower vertebrates (NACE, RICHARDS AND ASHER 1970).

MATERIALS AND METHODS

The materials and methods have been described in STREISINGER *et al.* (1981), CHAKRABARTI *et al.* (1983) and WALKER and STREISINGER (1983). Briefly, gametes were obtained by applying gentle pressure to anesthetized fish, and eggs were fertilized *in vitro*. To obtain homozygous fish, the eggs were fertilized with sperm that had been inactivated by UV irradiation and then subjected to either the late pressure (LP) or heat-shock (HS) procedure, which converts the haploid eggs to homozygous diploids by blocking the first cleavage. To obtain meiotic half-tetrads, the eggs were activated with UV-treated sperm and were then subjected to the early pressure (EP) procedure to block the second meiotic division. The nomenclature used for mutants was adapted from that for Caenorhabditis (HORVITZ *et al.* 1979).

RESULTS

The mutants: The appearances and origins of the mutants are summarized in Table 1. The pigment-pattern mutants *gol-1, gol-2, alb-1* and *spa-1* are easily recognized at 48 hr. The *alb-1* mutation is epistatic over the *gol-1* and *gol-2* mutations. *spa-1* mutations are difficult to recognize in young fish in the presence of the *alb-1* mutation.

Complementation: Each pigment-pattern mutation is recessive. To determine whether the mutations complement one another and to produce heterozygotes for segregation analyses, all possible pairwise crosses were performed. The F_1 progeny from each cross exhibited the standard type pigmentation, except for the cross of the golden mutants *b2* × *b3*, for which the F_1 progeny appeared indistinguishable from *b3* fish. The lack of complementation between *b2* and *b3* defines them as belonging in the same gene. The *b2* and *b3* mutant adults are golden in color; because they complement *gol-1* mutants and segregate independently from *gol-1*, they were assigned to a new gene *gol-2*.

Segregation analysis: To detect possible linkage between various mutants, the frequency of recombinants was counted among F_2 embryos which developed from LP- or HS-treated eggs of F_1 heterozygotes. The LP and HS treatments result in homozygosity (STREISINGER *et al.* 1981); thus, the phenotypically standard-type (*i.e.*, normally pigmented) fish in each cross are recombinants. Except for an uncertain case, there were no statistically significant departures for the frequencies observed for this class from the 25% expected for unlinked markers (Table 2). We conclude that no linkage exists among *gol-1, gol-2* and *alb-1*, and among *spa-1, gol-1* and *alb-1*. For crosses not involving *alb-1*, all recombinant and parental types were scored; all were approximately

TABLE 1

Description of mutants

Mutant[a]	Origin	Pigment pattern[b] at 2 days	Pigment pattern in adult
Standard type	Starting population	Large, black melanophores on body; pigmented retina in eye is black	Rows of large, black melanophores on body and on anal and caudal fins
gol-1(*b*1)	Present in starting population	No melanophores on body; no black pigment in eye	Rows of very small, black pigment cells; xanthophores are more prominent than in standard type
gol-2(*b*2,*b*3)	Obtained commercially	Large melanophores with light-brown pigment; pigmented retina in eye is brown	No black pigment; prominent rows of xanthophores; eye is deep ruby red
alb-1(*b*4)	Arose as a spontaneous mutant	No melanophores on body; no black pigment in eye	No black pigment; rows of xanthophores on body; more prominent leucophores than in standard type; eye is light red
spa-1(*b*5)	Present in *gol*-2(*b*2) stock	Large black melanophores on body, but fewer than in *gol*$^+$	Larger, but fewer, melanophores on body, arranged in less regular rows than in standard type; very high concentration of xanthophores in body and fins

[a] Gene names: *gol*, golden; *alb*, albino; *spa*, sparse.
[b] Pigment-bearing cells (chromatophores) mentioned in this table are melanophores (black or brown), xanthophores (yellow) and leucophores (white).

equal in frequency. For example, in the cross of *gol-1* and *gol-2*, the recombinants were found at frequencies of 0.25 and 0.27, and the parentals each at 0.24. The data for the cross between *spa-1* and *gol-1* were unclear. We confirm the lack of linkage between these two genes, and demonstrate the lack of linkage between *spa-1* and *gol-2* below.

Gene-centromere distances: To measure gene-centromere distances, we subjected eggs of heterozygous (F_1) females to the EP treatment (STREISINGER *et al.* 1981). This treatment blocks the second meiotic division. Before the EP treatment the eggs were activated by fertilization by sperm from which the genetic contribution had been eliminated by irradiation. Thus, the fish that develop from EP-treated eggs ("EP fish") have a diploid set of chromosomes that are derived from sister chromatids. They are half of a meiotic tetrad. F_1 females heterozygous for a particular recessive mutation produce two types of meiotic tetrads: (1) the sister chromatids are homozygous for the mutation or homozygous for the wild-type allele (Fig. 1, half-tetrads A and B), and (2) the sister chromatids are heterozygous for the mutation, because one chromatid has experienced an odd number of exchanges between the mutation and the centromere (Fig. 1, half-tetrads C and D). Progeny with mutant phenotypes

TABLE 2

Frequencies of segregants among F_2 progeny of crosses

Parents[a]		Progeny from LP- or HS-treated eggs					
I	II	Recombinant (standard type) [fraction (no.)]	Parental (I) [fraction (no.)]	Parental (II) [fraction (no.)] or Parental (II) and recombinant [fraction (no.)]	Recombinant (double mutant) [fraction (no.)]	(Total no.)	χ^2	Probability
gol-2	*gol-1*	(*gol-1*+ *gol-2*+) 0.25 (111)	(*gol-1*+ *gol-2*) 0.24 (106)	(*gol-1 gol-2*·)[b] 0.51 (225)		(442)	0.26	>0.7
				(*gol-1 gol-2*+) 0.24 (14)	(*gol-1 gol-2*)[c] 0.27 (16)	(30)	0.13	>0.5
gol-1	*alb-1*	(*alb-1*+ *gol-1*+) 0.22 (114)				(517)	2.48	>0.05
			(*alb-1*+ *gol-1*) 0.23 (32)	(*alb-1 gol-1*·) 0.56 (79)		(111)	1.09	>0.2
gol-2	*alb-1*	(*alb-1*+ *gol-2*+) 0.27 (112)	(*alb-1*+ *gol-2*) 0.24 (98)	(*alb-1 gol-2*·) 0.49 (199)		(409)	1.25	>0.5
spa-1	*gol-1*	(*gol-1*+ *spa-1*+) 0.20 (63)	(*gol-1*+ *spa-1*) 0.26 (81)	(*gol-1 spa-1*·) 0.53 (165)		(309)	3.52	>0.1
				(*gol-1 spa-1*+) 0.19 (28)	(*gol-1 spa-1*)[c] 0.34 (49)	(77)	5.73	<0.05
spa-1	*alb-1*	(*alb-1*+ *spa-1*+) 0.21 (89)	(*alb-1*+ *spa-1*) 0.26 (108)	(*alb-1 spa-1*·) 0.53 (218)		(415)	2.80	>0.2

Crosses were performed by fertilizing eggs of one mutant with sperm from another mutant. The F_1 progeny were raised to adulthood, and eggs of F_1 females were subjected to the LP or HS treatment. Most crosses are with fish derived from the starting population without preselection. Survival of homozygotes, therefore, was poor (STREISINGER *et al.* 1981).

[a] Because the parents and progeny of crosses were homozygous, the genotypes are described by single designations of alleles.

[b] Progeny were first scored at 2 and 3 days. At this time, certain genotypes could not be distinguished. A dot after the gene symbol (*e.g.*, *gol-2*·) indicates that the class includes both possible alleles (*e.g.*, *gol-2* and *gol-2*+).

[c] The class that contained fish of two different genotypes (footnote *b*) was scored again at a later time. The number examined was smaller than the original because only a fraction of homozygous fish survive. The fractions shown are relative to the total number of fish.

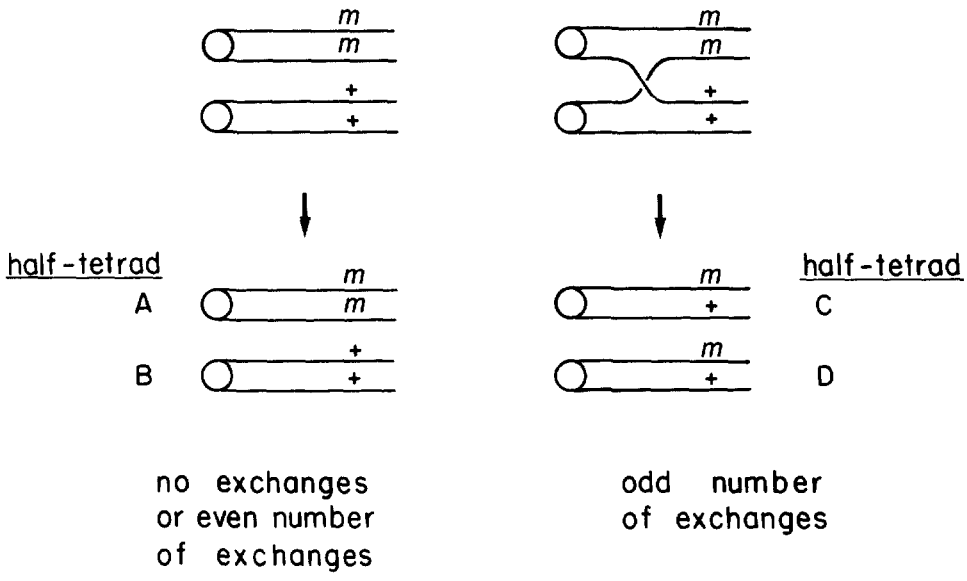

FIGURE 1.—Half-tetrad configurations.

TABLE 3

Gene-centromere distances

Mutation	Among progeny from EP-treated eggs			
	Fraction with mutant phenotypes (m)	Fraction of heterozygous half-tetrads ($1-2m$)	Gene-centromere distance: With complete interference[a]	Gene-centromere distance: From the HALDANE equation[b]
gol-1(b1)	0.056 (64/1151)	0.89[d]	44.5	110
gol-2(b2)	0.22 (128/595)	0.57	28.5	42
alb-1(b4)[c]	0.32 (585/1836)	0.36	18	22.5
spa-1(b5)	0.38 (113/299)	0.24	12	14
gol-1(b1)[c]	0.034 (42/1224)	0.93[e]	46.5	134
gol-1(b7)[c]	0.027 (19/716)	0.95[f]	47.5	146

[a] Fraction of heterozygous half-tetrads × 50.

[b] Calculated as $-(1n\ 2m) \times 100/2$, where m is the fraction of EP fish which exhibit mutant phenotypes.

[c] Measured in the inbred SPIII background; other measurements were with mutations in the starting population background.

[d,e,f] The differences due to background were significant (d vs. e: $\chi^2 = 10.4$; $P < 0.01$. d vs. f: $\chi^2 = 11.4$, $P < 0.001$). The difference between the two *gol-1* alleles in the same background was insignificant (e vs. f: $\chi^2 = 1.50$, $P > 0.2$).

are thus produced only from half of the sister chromatids that did not experience recombination between the mutation and its centromere (Figure 1, half-tetrad A). If m is the fraction of EP fish that exhibit mutant phenotypes, then the frequency of half-tetrads that are heterozygous for the marker of interest is $1-2\ m$. The frequencies of heterozygous half-tetrads range from 0.24 to 0.89 for the various genes examined (Table 3).

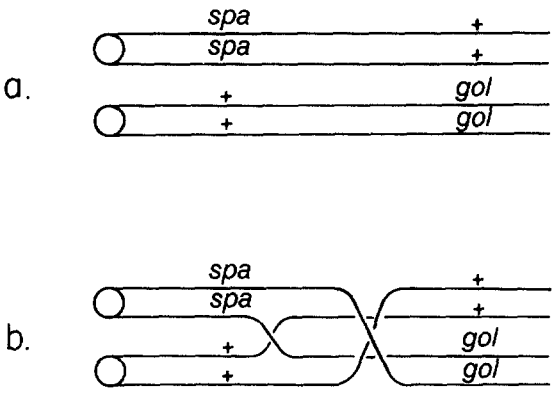

FIGURE 2.—Tetrad crossover configurations.

If exchanges are independent of each other, the maximum frequency of heterozygous half-tetrads is expected to be 0.67. The frequency of 0.89 for the *gol-1(b1)* mutation implies chiasma interference; that is, one crossover inhibits occurrence of another crossover in the same interval. Genetic distances between mutant sites and centromeres that have been calculated from the frequency of heterozygous half tetrads are given in Table 3, assuming either complete interference or no interference (HALDANE 1919).

Altered gene-centromere distances in different genetic backgrounds: The *gol-1(b1)* allele was present in our starting population of fish. A viable γ-ray-induced *gol-1* mutation [*gol-1(b7)* WALKER and STREISINGER 1983] recombined with the centromere with a higher frequency than did the standard *gol-1(b1)* mutation (the fraction of heterozygous half-tetrads being 0.95 and 0.89 for *b7* and *b1*, respectively; Table 3). The gene-centromere distance for the *gol-1(b7)* mutation was measured in the highly inbred SPIII strain, in contrast to the earlier measurements with the *b1* mutation which were obtained in the genetic background of the starting population. To determine whether the genetic background influenced the gene-centromere distance, the *gol-1(b1)* gene was transferred by repeated crosses into a stock nearly isogenic with SPIII fish. The frequency of recombinants is higher in the SPIII strain than in the starting population (0.93 *vs.* 0.89, Table 3). We conclude that the genetic background does influence the frequency of recombinants.

Segregation analysis using half-tetrads: Half-tetrad analysis provides for more sensitive tests of linkage than does the segregation analysis described in Table 2. For instance, consider the possible linkage of *spa-1* and *gol-1* or *gol-2*. Appearances of possible tetrads in an F_1 individual produced by crossing a *spa* and a *gol* mutant are shown in Figure 2. Progeny that exhibit a double mutant phenotype could arise only as a result of two exchanges in a recombinant ditype tetrad, as shown in Figure 2b. The occurrence of a double exchange would be expected to be relatively rare because of the chiasma interference mentioned earlier. As shown in Table 4, the observed frequencies of fish with double mutant phenotypes are those expected on the basis of independent assortment (Table 4, compare column 5 with 6 and column 7

TABLE 4

Linkage analysis using half-tetrads

Parents	Among F_2 progeny from EP-treated F_1 eggs									
	No. sparse and golden	No. sparse[a]	No. golden[a]	Total no.	Fraction golden among sparse	Fraction golden among total	Fraction sparse among golden	Fraction sparse among total	χ^2	Probability
	spa gol / *spa gol*	*spa* / *spa gol*·	*spa*· / *gol gol*							
	(1)[c]	(2)	(3)	(4)	(5)=	(6)=	(7)=	(8)=		
gol-1 spa-1	9	257	20	710	0.035	0.028	0.45	0.36	0.24	>0.1
gol-2 spa-1[b]	28	113	72	299	0.25	0.24	0.39	0.38	0.003	>0.9

[a] A dot after the gene symbol indicates that the class includes both possible alleles.
[b] The population of fish described here is the same as the one used for measuring the distance of *spa-1* from its centromere.
[c] Numbers in parentheses denote column numbers.

with 8). These results confirm the lack of linkage between *gol-1* and *spa-1*, and establish the lack of lineage between *gol-2* and *spa-1*.

DISCUSSION

The production of homozygotes greatly facilitated the measurement of recombination frequencies in zebrafish, We have shown that the *gol-1, gol-2, alb-1* and *spa-1* genes are not closely linked, validating the use of these as independent markers in mutagenesis experiments (CHAKRABARTI *et al.* 1983).

The frequencies of recombinants were not significantly different from the expectations for random segregation. In the cross *gol-1* × *spa-1*, the frequency of the gol^+ spa^+ recombinant class was lower than 25% (20%). This result by itself might have suggested linkage, but the observation that the reciprocal recombinant class was higher than 25% (34%) made linkage improbable. The departures from randomness could be due to statistical fluctuations or to the presence of deleterious mutations present in the starting population and linked to one of the markers. Crosses using homozygous (and, thus, lethal-free) fish will exclude the latter possibility. Lack of close linkage of *spa-1* to *gol-1* was confirmed by half-tetrad analyses.

The observation of >67% tetratype (*i.e.*, second-division segregation) tetrads is not unique to zebrafish; >67% second-division segregations have been observed in many organisms, including trout (THORGAARD, ALLENDORF and KNUDSEN 1983), Neurospora, yeast and Drosophila, where they have been ascribed to chiasma interference (see PERKINS 1955). Two limits are presented in Table 3 for relative map distances from gene to centromere; one based on complete interference (that is, only one crossover allowed in an interval) and one based on no interference. The latter is derived from the classical HALDANE (1919) equation: recombination frequency = $\frac{1}{2}(1-e^{-2x})$, where x is the map distance/100. Because of the interference observed, the relative genetic map distances are expected to be close to the values calculated on the basis of complete interference. The best estimates for physical distance depend partly on the mechanisms responsible for interference. The interpretation of gene-centromere distance is of particular interest because of appreciable decreases that we have observed in this distance for certain γ-ray-induced *gol-1* mutations, which might be extensive deletions. Comparisons of calculated relative distances for these possible deletion mutations with cytological measurements will be informative.

The observed differences in gene-centromere recombination frequencies in different genetic backgrounds are not surprising. Although the differences we observe in different genetic backgrounds are statistically significant, they need to be confirmed for several different markers in order to be interpretable. The differences could be due to altered chromosome length, altered recombination machinery or alterations in the degree of chiasma interference, to name but a few possibilities.

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