

Diploid-dependent regulation of gene expression: a genetic cause of abnormal development in fish haploid embryos

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A diploid-dependent regulatory mechanism of gene expression for spatial patterning of the eye in vertebrates has been determined by analyzing the phenotypes of haploid goldfish embryos. There are two gene loci in charge of eye spatial patterning during embryonic morphogenesis. The expressional probability for each copy of the two genes in a set of chromosomes is 50%. A pair of genes in two sets of

homologous or heterologous chromosomes is 100% and essential for normal gene expression. The haploid condition itself would result in the obstruction of gene expression and abnormal development because the diploid-dependent regulatory apparatus will regulate gene expression in a haploid embryo according to the same rule as in the diploid embryo. *Heredity* (2003) 90, 405–409. doi:10.1038/sj.hdy.6800263

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Introduction

The animal developmental program proceeds in a given precise pattern of space and time. This spatial and temporal pattern is controlled by a strikingly complex genomic regulatory system. Investigating the genomic regulatory system and mechanism are of fundamental importance for understanding both development and evolutionary bioscience.

Considerable investigation of the molecular basis of development has concentrated on individual genes' promoters and their various regulatory elements. However, the genomic regulatory system and general principles at the whole genome level have remained frustratingly elusive in the vertebrate. This is largely because of its extreme complication, the absence of an appropriate sample, and the method of experimental analysis.

According to genetic theory, the genes in one set of homologous chromosomes should be sufficient to encode all the structures and physiological functions of an individual. However, all of the artificially induced haploid embryos of amphibians (Hertwig, 1911; Fankhauser, 1945) and fish (Oppermann, 1913; Purdom, 1969; Nagy *et al.*, 1978; Chourrout, 1980; Streisinger *et al.*, 1981; Refstie *et al.*, 1982; Taniguchi *et al.*, 1986; Komen *et al.*, 1991; Luo and Liu, 1991; Varadi *et al.*, 1999; Galbusera *et al.*, 2000) have shown abnormalities and have died during embryonic development. The abnormality and mortality of haploids has generally been assumed to be because of the presence of deleterious recessive lethal genes in the haploid genome (Fankhauser, 1945; Purdom, 1969;

Thorggard, 1983). Although such recessive lethal alleles may contribute to the death rate, they do not appear to be the underlying problem. In special conditions, diploid mitotic gynogenes from eggs obtained from the same single spawning survive well and do not show the same untimely death of the haploids (Chourrout, 1982; Mair *et al.*, 1987). This phenomenon suggests that the haploid syndromes and high mortality must be due more to the lack of homologous genes in pairs rather than being due to the presence of lethal alleles (Varadaraj, 1993). In other words, the haploid itself might result in the obstruction of gene expression in embryonic development in the vertebrate. Obviously, analyzing the phenotypes of haploid and diploid embryos, which share the same genetic background but differ in the number of sets of chromosomes, would provide a new method for investigating regulation of gene expression at the whole genome level.

Of all the organs of the vertebrate body – apart from the brain – the eye is the most complex, particularly in terms of its cell diversity, component tissues, and parts, which are united to form an optic instrument of amazing proficiency and efficiency. Also, the eye of the vertebrate is the organ that has been studied in greatest depth. The complex course of development has been fairly well worked out using classical embryological methods (Ston, 1960; Lopashov and Stroeveva, 1961; Jacobson, 1966; Bloemendal, 1977; Turner and Cepko, 1987; Saha *et al.*, 1989). All of the differentiated cells and parts of the eye arranged in a given spatial pattern can be identified easily. Recently, more than 29 transcription factors required for the development of the vertebrate eye have been identified (Jean *et al.*, 1998; Haider *et al.*, 2000; Horsford *et al.*, 2001; Chow *et al.*, 2001) and several mutations affecting development of the retina in zebrafish (Malicki *et al.*, 1996; Malicki and Driever, 1999; Jensen *et al.*, 2001; Vihtelic and Hyde, 2002) have been detected.

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Therefore, the morphogenesis of the eye is suitable for an experimental analysis to study the genetic causes of abnormality in haploid embryos of vertebrates.

Materials and methods

Animals

A white, a red, and a red cup inbred strain of goldfish, *Carassius auratus*, obtained from a goldfish-breeding farm, were employed in this study. In each experiment, all the eggs used both in tests and in controls were obtained from a single spawning. Common carp, *Cyprinus carpio*, were obtained from a local fish farm.

Production and identification of gynogenetic haploid embryos

Haploid gynogenes of the goldfish were induced using a UV-irradiated sperm of common carp (Luo and Liu, 1991). The goldfish has a specific double tail encoded by a recessive gene, while the common carp has a common single tail encoded by a dominant allele. The tails of all the hybrid embryos of the goldfish and common carp are all single tails just like the tail of the parent common carp (Luo, unpublished observations). With this character, the gynogenetic haploid embryos could be identified easily and unequivocally from the normal and abnormal hybrid embryos at an early developmental stage.

In inbreeding and intergeneric hybrid diploid control groups, the eggs coming from the same spawning were fertilized with normal sperm coming from the same inbred goldfish line or common carp, respectively.

Production and identification of mitogynogenetic diploid embryos

The optimum parameters for chromosome set duplication in goldfish were determined by systematical cytological observation on the developmental procedure

of the first cleavage with the method of histological section (Li *et al*, unpublished result). After being stimulated to start development by UV-irradiated sperm, the activated eggs of goldfish were reared at 18°C. Mitogynogenetic diploid embryos were produced by inhibiting the first mitosis of the activated haploid eggs around 40 min after activation with a heat shock of 40°C applied for 2 min. The haploid embryos can be distinguished from the diploid gynogenetic embryos by their characteristic syndromes such as twisted edematous body and curved tail. The mitogynogenetic homozygous diploids could be identified easily and unequivocally from the normal and abnormal hybrid diploid embryos at an early developmental stage by their tails.

Results

Some gynogenetic haploid embryos of the goldfish could develop to the hatching stage. However, none of them survived beyond the feeding stage. No diploid embryo with double tail was observed in any of the tests in that the eggs were activated with UV-irradiated common carp sperm but not treated with heat shock. In general, almost all the organs showed abnormality in a gynogenetic haploid group, although some organs of a given individual of the gynogenetic haploid group appeared normal.

Abnormality of eyes of the haploid embryos could be identified easily by specific defects in the retinal pigmented epithelium (Figure 1a) at an early stage. Normal and abnormal eyes were all observed in gynogenetic haploid embryos. Both eyes of a given haploid individual were either normal or abnormal. No haploid individual with one normal eye and one abnormal eye was observed. Some differences among the abnormal eyes were observed. However, the differences of abnormalities could not be distinguished distinctly. Therefore, the abnormal eyes were not further

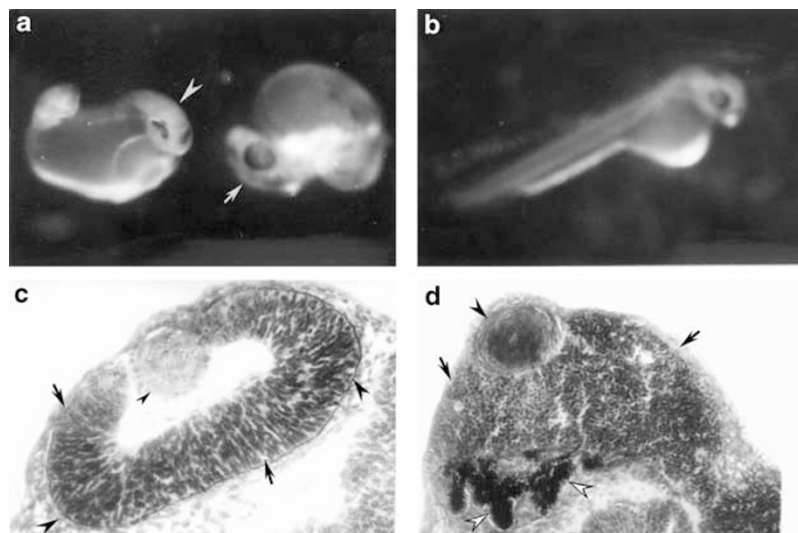


Figure 1 Normal and abnormal eyes of haploid goldfish embryos at hatching stage. (a) Haploid embryos with abnormal (arrowhead) or normal (arrow) eyes; for a given haploid embryo, the two eyes are either both normal or both abnormal. (b) A diploid embryo of control. (c) Microstructure of a normal eye of a haploid embryo, the differentiated cells, proportions, and the spatial pattern are just the same as that of the eye of the diploid embryo. Retinal pigmented epithelium: bigger arrowheads; neural retina: arrows; lens: smaller arrowhead. (d) Microstructure of an abnormal eye of a haploid embryo, the differentiated cells and proportions of the eye are not arranged in a correct spatial pattern. Retinal pigmented cells: white arrowheads; neural retina cells: black arrows; lens: black arrowhead.

Table 1 Number of embryos with normal or abnormal eyes in gynogenetic haploid, mitogynogenetic diploid, inbred diploid, and intergeneric hybrid diploid

Strains	Type of embryos	Total number	Embryos with abnormal eye	Embryos with normal eye	Ratio of normal and abnormal eyes
Red	Gynogenetic haploid	802	605	197	1 : 3.02
	Mitogynogenetic diploid	60	0	60	
	Inbred diploid	358	0	358	
	Intergeneric hybrid diploid	336	0	336	
White	Gynogenetic haploid	419	303	116	1 : 2.60
	Mitogynogenetic diploid	48	0	48	
	Inbred diploid	356	0	356	
	Intergeneric hybrid diploid	425	0	425	
Red cup	Gynogenetic haploid	3046	2255	791	1 : 2.85
	Inbred diploid	318	0	318	

All the haploid and diploid embryos were from eggs obtained from a single spawning in each test and were counted at the hatching stage. Haploid embryos were identified using both the characteristic syndromes of haploids and the double tail of goldfish. Abnormality of the eye was identified according to the characteristic defects of the retinal pigmented epithelium.

classified into subgroups and the variation was not further analyzed.

The results of a statistical analysis of the haploid individuals with normal or abnormal eyes shown in Table 1 are very surprising. In every test, about one-fourth of the haploid gynogens developed normal eyes, the other three quarters developed obviously abnormal eyes. However, in the controls of mitogynogenetic diploids, inbred diploids, and intergeneric hybrid diploid embryos, no specific abnormal eyes, as seen in the haploid embryos, were observed in any of the tests.

On the histological sections of the embryonic eyes, the microscopic structure of the normally developed eyes of the haploid embryos was just like the structure of the control diploid embryos at the same stages (Figure 1c). The abnormal eyes of the haploid embryos had also developed all of the differentiated cellular types and parts of the vertebrate eye, such as pigment retina cells, neural retina cells, lens, and cornea. However, these differentiated cells or parts were not arranged in the correct positions (Figure 1d). No optical cup was formed on any of the observed abnormal eyes of the haploid gynogenes. The pigmented cells, which should be distributed evenly on the whole outer layer of the optical cup and form the retinal pigmented epithelium in the normal eye, assembled in a small region. The neural retina cells, which should constitute the inner layer of the optical cup, were in a disordered state. There was no cup between the lens and the neural retina cells in any of the abnormal eyes examined from haploid embryos. These results indicated that the abnormal development of the eye in haploid embryos did not result from abnormal differentiation but from an abnormal positional arrangement of the differentiated cells and parts.

Discussion

Using two different species of fish that have distinct genetic marks, the possibility of paternal contamination can be excluded. Meanwhile, all the gynogenetic haploid embryos had died at the first-feeding stage and no spontaneous gynogenetic diploid individual has been

observed. These observations suggest that meiosis is normal in these fish, and the gynogenetic offspring could not be a mixture of mitogynogenetic homozygous and spontaneous meiotic heterozygous individuals.

The observed ratio of about one to three has revealed that, during the development of the eye in the goldfish, there are two different genes in charge of the spatial positional arrangement of the differentiated cells and parts. Since there is only one set of chromosomes in haploid embryos, if there were two allelic genes, one dominant wild type and the other a deleterious recessive mutation, the ratio of haploid embryos with normal and abnormal eyes would be about one to one. Therefore, any two genes involved are not alleles of the same genetic locus but different alleles at two gene loci. Since no abnormal eyes appeared in either the controls of mitogynogenetic diploid embryos or by inbreeding diploid embryos that shared the same genetic background as the haploid embryos, it is impossible that there could have been deleterious recessive alleles of both gene loci in all of the three inbred strains. The normal and abnormal phenotypes of the eye therefore do not reflect genotype, but rather, the expressional state of the two genes. It appears that some obstruction of gene expression in the haploid has resulted in an abnormal spatial pattern of the eye.

Consequently, the ratio of about one to three also indicates that each copy of the two regulator genes in a set of chromosomes only has a 50% chance of being selected for expression, both in haploid and diploid embryos. In diploid embryos, the combined expression probability for a pair of genes is 100%. Therefore, there would be only one composite type of gene expression that both genes express and all the eyes are normal. In haploid embryos, there would be two possible expression states for a gene. That is to say, that a gene could be either expressed or silent. Since the eye spatial patterning is arranged by two genes as reasoned above, there would be four possible composite expression types according to mathematical logic (Figure 2). Only the type in which both the two genes are expressed would develop normal eyes. The probability that the two genes would be simultaneously expressed is one-fourth of the haploid

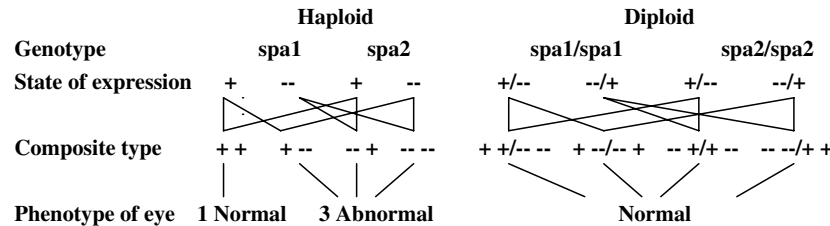


Figure 2 Expression of two spatial arranging genes (*spa*) and relative phenotypes of eyes in haploid and diploid embryos of goldfish. The two genes in charge of spatial pattern arrangement of the eye were named *spa1* and *spa2*. There was 1/2 probability for every copy of the two genes in a set of chromosomes to be selected for expression both in haploids and in diploids. In haploid conditions, single copy of a gene is either in expression (+) or in silence (-) condition by chance. Therefore, there would be four different composite types in terms of gene expression (++, +-, -+, --) for the two genes in haploid embryos. Only one type in which the two genes, both were selected for expression (++) would develop normal eyes. The other three-fourths would develop abnormally for either one (+-, -+) or both (--) of the two genes were not selected for expression. In diploid conditions, there was a 100% probability for the pair of genes to be selected for expression (+/- or 1-/+). Therefore, there would be only one composite type where both genes are selected for expression and all the diploid embryos develop normal eyes.

embryos. As a result, there would be only one-fourth of the haploid embryos that develop normal eyes. The other three types of haploid embryos develop abnormal eyes because at least one of their two regulator genes was not expressed.

The results suggest that, in embryonic morphogenesis of vertebrates, (1) there are some genes responsible for the spatial patterning, and (2) the regulatory mechanism of expression of these spatial-control genes is not based on one but two sets of chromosomes. Vertebrates have established an ingenious regulatory apparatus to modulate transcription of the genes on the two sets of chromosomes in a precisely logical manner during evolution. In haploid, the diploid-dependent regulatory apparatus selects a gene for expression according to the same rule as in diploid, which results in some genes not being selected for expression. This may be the cause why haploidy itself can result in obstruction of gene expression and developmental abnormality, at least of the spatial pattern formation of the eye in vertebrates.

Recently, Jensen *et al* (2001) have isolated two mutations of the *mosaic eyes* gene in which the retinal organization is severely disrupted. The mutations of the *mosaic eyes* gene cause retinal pigmented epithelium abnormalities, retinal abnormalities, reduced brain ventricles, circulation abnormalities, and dorsal curvature of the tail, but do not cause abnormal retinal cell differentiation. These phenotypes are similar to the phenotypes of those haploids. We reason that the *mosaic eyes* gene might be one of the spatial patterning control genes affected in the haploids.

The diploid-dependent regulatory apparatus and its nature have not yet been fully investigated. The reason why the regulatory apparatus modulates gene expression according to precise mathematical logic, but cannot distinguish the haploid and diploid conditions is very confusing and interesting.

One possible explanation might be based on imprinting of DNA methylation. In mammals, it has been demonstrated that the presence of methylated cytosines in the promoter of a gene correlates with the repression of transcription from that gene (van der Ploeg and Flavell, 1980; Groudine and Weintraub, 1981; Bussslinger *et al*, 1983). The pattern of methylation on a given gene can differ between egg and sperm (Barlow *et al*, 1991; DeChiara *et al*, 1991; Bartolomei and Tilghman, 1997).

This maternal imprinting may be responsible for the abnormal development of the gynogenetic goldfish haploids, since some genes, inherited from the mother, are switched off by methylation. In this case of observations, two unlinked gene loci being required in active form for eye development, one could imagine that, in the mother's meiosis, homologous pairs of the genes at both loci have one of their alleles switched off by methylation, and, the alleles then segregate into the ovum. Therefore, a haploid embryo would only have a one-in-four chance of receiving the active alleles at both loci. However, such a model would raise questions as to why diploids always have at least one active gene copy. One hypothesis could be that the repression of the female's genome by methylation is removed at the moment of fertilization. However, in the case of all the eggs used in each experiment being obtained from a single spawning, if the methylation is erased at fertilization, the maternal imprinting introduced onto the genome during meiosis should be the same. Therefore, this hypothesis would raise a second question as to how the females could have one but only one inactivated copy of some genetic loci. We further hypothesize that only if all the imprinting of the maternal genome is removed at fertilization, the methylation information imprinted on the paternal genome remain so throughout development, and are passed on in their imprinted state by their daughters. The hypothesis would also raise a third question as to why the maternal-inherited methylation information is not erased in induced gynogenetic haploids. A third hypothesis is required: the activation effect with UV-irradiated sperm could not have the effect of removing the repression of maternal-inherited alleles that is postulated to occur at fertilization.

The diploid-dependent regulatory apparatus and its nature might have something to do with the *cis*-regulatory system. A very interesting promoter that operates in a logical manner has been reported (Arnone and Davidson, 1997; Yuh *et al*, 1998). Experimental and computational analysis revealed that the logical functions of the *Endo16 cis*-regulatory system are all dependent on explicitly specified DNA sequences in the genome. Perhaps the vertebrates also have evolved specified DNA in their genome for mediating the diploid-dependent regulatory apparatus to process in logical manner.

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