Characterization of transgene integration pattern in F4 hGH-transgenic common carp (*Cyprinus carpio* L.)

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ABSTRACT

The integration pattern and adjacent host sequences of the inserted pMThGH-transgene in the F4 *hGH*-transgenic common carp were extensively studied. Here we show that each F4 transgenic fish contained about 200 copies of the pMThGH-transgene and the transgenes were integrated into the host genome generally with concatemers in a head-to-tail arrangement at 4-5 insertion sites. By using a method of plasmid rescue, four hundred copies of transgenes from two individuals of F4 transgenic fish, A and B, were recovered and clarified into 6 classes. All classes of recovered transgenes contained either complete or partial pMThGH sequences. The class I, which comprised 83% and 84.5% respectively of the recovered transgene copies from fish A and B, had maintained the original configuration, indicating that most transgenes were faithfully inherited during the four generations of reproduction. The other five classes were different from the original configuration in both molecular weight and restriction map, indicating that a few transgenes had undergone mutation, rearrangement or deletion during integration and germline transmission. In the five types of aberrant transgenes, three flanking sequences of the host genome were analyzed. These sequences were common carp *β-actin* gene, common carp DNA sequences homologous to *mouse phosphoglycerate kinase-1* and *human epidermal keratin 14*, respectively.

Keywords: transgenic common carp, plasmid rescue, germline transmission, integration pattern.

INTRODUCTION

After the development of the transgenic "super mouse" by introduction of a novel growth hormone gene construct into mice fertilized eggs [1], gene transfer has been extensively studied in numerous species [2]. The integration, expression and germline transmission of transgenes is the prerequisite of animal transgenesis [3]. In fish embryos, since the novel gene is commonly injected into the egg cytoplasm and the early embryonic cell cleavage is fairly rapid, the integration of foreign gene occurs during embryogenesis, resulting in transgenic mosaics [4, 5]. The germline transmission of transgenes in fish has been described by different authors [6–9]. However, little is known about the transgene's integration sites and stability or integrity in the host fish following reproduction.

In a tested model, transgenic common carp were produced by microinjecting a recombinant plasmid pMThGH into common carp fertilized eggs. To keep the genetic diversity of transgenic offspring and to combine different valuable traits of transgenic founders, we produced the transgenic F1 offspring by crossing one transgenic male with four transgenic females. The F4 pMThGH-transgenic common carp was raised through hybridization between transgenic males and transgenic females generation by generation. In F4 generation, 100% of the offspring contained pMThGH-transgene and displayed normal expression of transgene [10]. Compared with the controls, the F4 transgenic common carp have improved growth rate and feed utilization efficiency, just like the transgenic founders [11]. In addition, we obtained seven cross-genus cloned fish derived from F4 transgenic common carp nuclei and goldfish eggs [12]. However, up to the present, comprehensive information about the status of the transgene copies in F4 transgenic fish has been lacking. Since the technique of plasmid rescue was firstly described by

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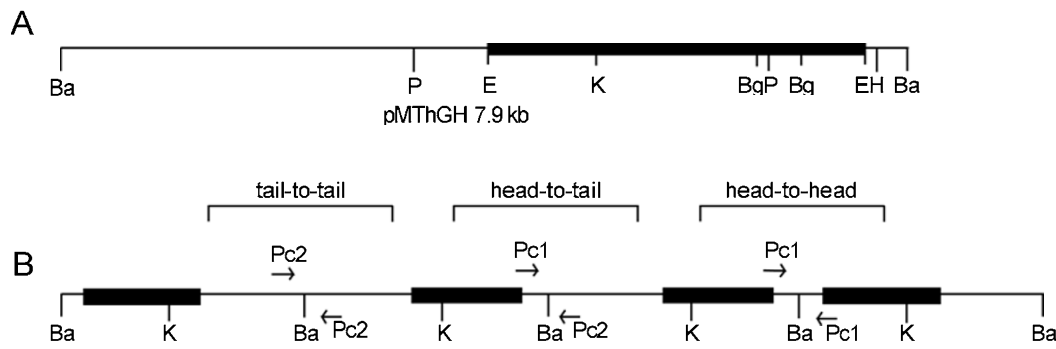Tel: +86-27-6878 0628; Fax: +86-27-6878 0628;
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Fig. 1 The plasmid pMThGH and sketch map of various joint transgenes. (**A**) Physical map of pMThGH. Thick line refers to *MThGH* gene. Fine line is the vector sequence of pBR322. Ba, *Bam*HI; E, *Eco*R I; H, *Hind*III; P, *Pst*I; K, *Kpn*I; Bg, *Bgl*II. (**B**) Different types of joint transgenes. Pc1 and Pc2 refer to the primers used for PCR detection of the joint transgenes.

Perucho *et al* [13]), it has been utilized in the study of transgenic mice [14–17], transgenic tomato [18] and transgenic *Drosophila* [19], but it has rarely, to our knowledge, been employed previously in the study of transgenic fish. Recently, we briefly reported three integration-site sequences in F4 transgenic fish [20]; however, the detailed integration pattern of transgene in F4 transgenic fish needs to be clarified. Here we adopted the method of plasmid rescue to study the copy number, manner and sites of transgene integration in the F4 pMThGH-transgenic fish. This study will provide a comprehensive understanding of the integration pattern and the inheritance stability of transgenes after four-generation transmission in transgenic fish.

MATERIALS AND METHODS

Production of F4 pMThGH-transgenic fish

A recombinant DNA fragment of 3.5 kb, containing a mouse metallothionein-1 (*MT*) promoter and regulation region and human growth hormone "mini-gene" (*hGH* with introns 2, 3 and 4 deleted), was cloned into pBR322 at the *Eco*RI site. The resulting plasmid, pMThGH (Fig. 1A), was digested by *Bam*HI and microinjected into the fertilized eggs of common carp to produce the transgenic founders [4]. After being confirmed by dot blotting and PCR, a female transgenic founder with significant growth enhancement was mated with 4 male transgenic founders and the pool of F1 offspring was produced. The transgenic ones of F1 offspring were mated each other to produce the F2 generation, and the F3 generation was subsequently produced by random mating among the transgenic ones of F2 offspring. A pair of transgenic F3 individuals was mated to produce the F4 offspring (Fig. 2). Two heaviest individuals (named as fish A and B) of the F4 fish in one-year age were sampled and used in the present study.

Dot blotting and PCR analysis of transgene in host fish

High-molecular-weight genomic DNA was isolated from tail fin of the F4 fish as described [4]. In order to identify the copy number of the transgene in F4 fish, dot blotting was carried out with 10 μg

genomic DNA of each fish against DIG-labeled pMThGH probes (DIG DNA Labeling and Detection Kit, Roche). Quantity control was equally carried out using ten-fold serially diluted pMThGH DNA, with the initiative quantity of 50 ng. Primer labeling, dot blotting and detection were performed according to the user's manual. PCR primers were designed to determine concatenate status of transgene in host fish. The sequences were Pc1 (5'-GTTAAATTG-CTAACGCAGTCAGGC-3') and Pc2 (5'-TGATGTCGGCGATAT-AGGCG-3'), respectively. Three combinations of primers were used for PCR amplification: Pc1 alone, Pc2 alone and Pc1+Pc2. If the concatenates were formed in a head-to-head manner, Pc1 alone would give specific PCR amplification; if in a tail-to-tail manner, Pc2 alone would give specific amplification; if in a head-to-tail manner, Pc1+Pc2 would give specific amplification. The expected amplification length of Pc1 alone is 643 bp, Pc2 alone is 159 bp and Pc1+Pc2 is 401 bp (Fig. 1B). PCR reaction parameters were as follows: 1 U of Taq DNA polymerase (MBI), 400 μM of each primer, and 100 ng of genomic DNA in a total volume of 25 μl; 94°C, 4 min, 30 cycles of 94°C, 30 sec; 60°C, 30 sec; and 72°C, 1 min.
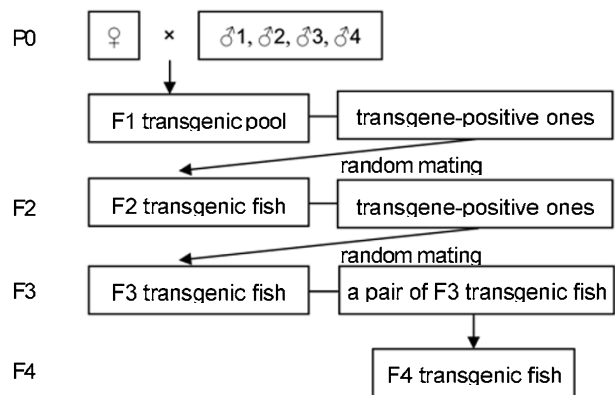

Fig. 2 Diagram of the breeding history of F4 transgenic fish.

Genetic analysis of the F4 transgenic fish

In the two series, fish A and B were each artificially fertilized with the sperm from non-transgenic common carp. For each series, 100 hatched fry were randomly selected for extraction of total DNA. Total DNA was extracted and PCR assay was carried out to detect the presence of the transgene in hatched fry. Sense primer P_+ (5'-GGTAAGCGCCCCTAAAATCC-3') was located across the end of exon 1 and the beginning of intron 1 and the anti-sense primer P_- (5'-TTGAAGATCTGCCCAGTCCG -3') was at the exon 2 of the *hGH* mini-gene. The length between the two primers on the *hGH* mini-gene was 747 bp. PCR reactions consisted of 30 cycles: 30 sec at 94 °C, 30 sec at 58 °C, 1 min at 72 °C, with a 2 min initial 94 °C denaturation and a 5 min final 72 °C elongation. Based on the transgene positive ratios in two series, the number of integration sites in each F4 transgenic fish was deduced according to the Mendel's law. Statistical analysis was carried out as goodness-of-fit test for discrete random variables.

Recovery of the pMThGH-transgene

Transgenes were recovered by a modified plasmid rescue technique [21]. 10 μg of the genomic DNA was partially digested with 4 U *Bam*HI (Promega) in 100 μl total reaction volume. At time interval of 2, 5, 10, 15, 30 min, every 1/5 volume of the reaction was collected and the reaction was stopped by addition of EDTA to a final concentration of 0.05 M. Digested DNA fragments ranging from 4 to 12 kb were recovered and purified with glassmilk (Biostar). The recovered DNA fragment in 1.8 μg/ml was self-circularized with 3 U of T_4 DNA ligase (Promega) at 16 °C overnight. The circularized DNA was transformed into competent cells of *E. coli* Top10F strain. Transformed cells were spread onto LB plates containing 50 μg/ml ampicillin. The circularized pUC19 DNA was transformed as a positive control. In addition, *Kpn*I digested genomic DNA was circularized and transformed into *E. coli* as well.

Classification, mapping, Southern hybridization and PCR of recovered transgenes

Plasmid DNA of each clone of the recovered transgene was prepared and doubly digested with *Eco*RI and *Bam*HI for classification. The classified clones are named as fish A- and fish B- series. Physical mapping of the clones was carried out with two groups of double digestion of the plasmid DNA: (1) *Bam*HI plus one of *Eco*RI, *Bgl*II, *Pst*I, *Kpn*I or *Hind*III, and (2) *Hind*III plus one of *Eco*RI, *Bgl*II, *Pst*I or *Kpn*I. Based on the restriction maps, each class of the transgene DNA was digested with appropriate enzymes for Southern hybridization: A1, A2, B1 and B2 were digested with *Bam*HI and *Eco*RI; A3 was digested with *Pst*I; A4 and B3 were digested with *Bam*HI; A5 and B4 was digested with *Hind*III and *Bgl*II; A6 was digested with *Hind*III and *Eco*RI. Southern hybridization of the digests was performed against DIG-labeled pBR322-absent *MThGH* probe. PCR analysis of the recovered transgenes was carried out as described previously.

Characterization of host genome DNA adjacent to the recovered transgenes

Based on the restriction map and Southern DNA hybridization, host DNA fragments adjacent to the transgene were deduced and subcloned into pUC19. DNA sequences of these fragments were determined using the dideoxy sequencing method with M13 universal primers. The data collection was automatically performed on
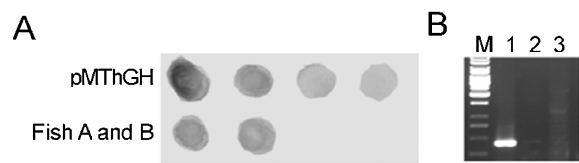

Fig. 3 Copy number and concatenation of transgene in host fish. (**A**) The upper series refer to dot blotting against serially diluted pMThGH DNA of ten-fold with an initiative quantity of 50 ng. The lower series refer to dot blotting against 10 μg of genomic DNA of fish A and B. (**B**) PCR results of transgenic fish with the primers Pc1 and Pc2. M: molecular marker 1kb DNA ladder (MBI co.); 1, Pc1+Pc2; 2, Pc1 alone; 3, Pc2 alone.

the ABI 310 Genetic Analyzer (PE Applied Biosystems). According to the sequence data of these fragments, three sets of primers were designed for amplification of the supposed adjacent DNA fragments among F4 transgenic fish and non-transgenic fish. The primer sequences are listed as follows: for fragment 1, 5'-GAATTCTAC-CGGGTAGGGGA-3' and 5'-TATCTAATCCCACCCCACCC-3'; for fragment 2, 5'-GGATGGATACCCGGCTGGAA-3' and 5'-TTGGGGCTAAGCCTGGGCTA-3'; for fragment 3, 5'-GCCACT-AAATCACACTGTCCTTGG-3' and 5'-CTGCAGTCACTT-CAGCGACTCTT-3'. The PCR amplification reactions were conducted with parameters similar to those for transgene detection, but with PCR cycles of annealing temperature of 55°C and elongation time of 2 min.

RESULTS

Copy number and concatenation of transgene in host fish

The result of dot blotting showed that the signal density of two individuals of F4 fish was indicative of 5 ng pMThGH (Fig. 3A). Since the genome size of common carp haploid genome is about 1.7 pg [22], the number of diploid genome equivalent in 10 μg of common carp genomic DNA can be calculated as 10 μg/ 1.7 pg/2 = 3×10^6. Since the size of the plasmid pMThGH is 7.88 kb and 1 copy of 1 kb DNA molecular weights about 10^{-6} pg, it can be calculated that 5 ng pMThGH contains 6.3×10^8 (5 ng/ 7.88×10^{-6} pg) copies of the plasmid molecules. It can therefore be estimated that each genome of the F4 fish contains approximately 200 ($6.3 \times 10^8/3 \times 10^6$) copies of transgene.

In the three combinations of PCR analysis with the primers Pc1 and Pc2, Pc1+Pc2 produced the specific amplification band of 401 bp (Fig. 3B), while the other two combinations (Pc1 or Pc2 alone) did not produce any specific amplification. Since the injected plasmid was linearized with *Bam*HI, those plasmids linked in a head-to-tail manner could be amplified merely with the primers Pc1 and Pc2 (see Fig. 1B). If the injected plasmids were linked in a head-to-head or tail-to-tail manner, Pc1 or Pc2

alone will give specific amplification. The results revealed that pMThGH transgenes were tandemly arrayed with a head-to-tail manner in host fish, resulting in transgene concatemers in the F4 genome.

Insertion numbers of transgenes in F4 transgenic fish

In two series of mating experiments from fish A and B with non-transgenic fish, the positive ratios of pMThGH in the hybrid fry were each 96% and 94%. According to the Mendel's law, if there is only one transgene insertion in each fish, the positive ratio will be 50%; if there are one

Tab. 1 Types of transgene colonies recovered from fish A and B.

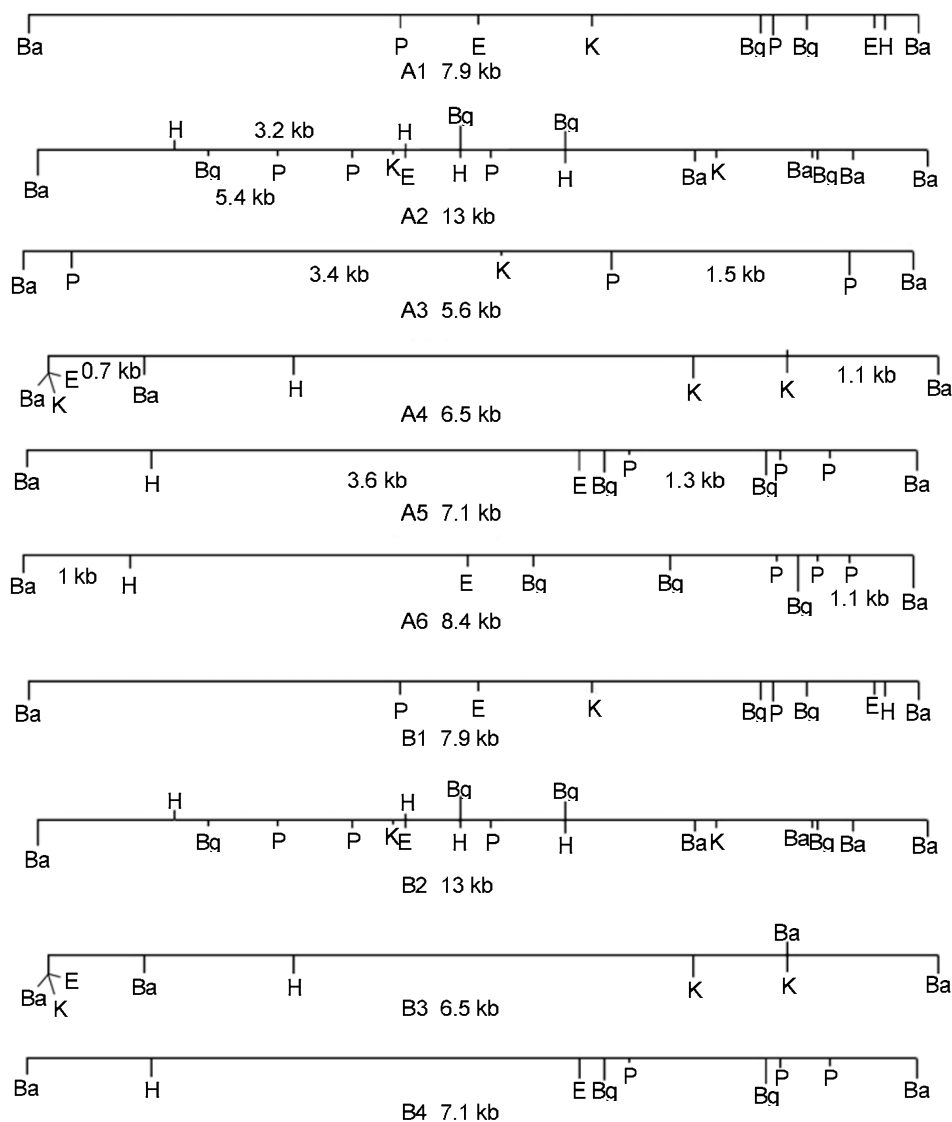| Fish A	Fish B
A-1, 7.9 kb (166 in total, 83%), the electrophoretic pattern was the same as that of pMThGH	7.9 kb (169 in total, 84.5%), the electrophoretic pattern was the same as that of pMThGH
13 kb (1 in total, 0.5%)	13 kb (3 in total, 1.5%)
5.6 kb (3 in total, 1.5%)	6.5 kb (27 in total, 13.5%)
6.5 kb (26 in total, 13%)	7.1 kb (1 in total, 0.5%)
7.1 kb (3 in total, 1.5%)	
8.4 kb (1 in total, 0.5%)	

Fig. 4 Restriction analysis of six types of recovered plasmids from fish A and four types of recovered plasmids from fish B. Ba, *Bam*HI; E, *Eco*RI; H, HindIII; P, *Pst*I; K, *Kpn*I; Bg, *Bgl*II.

or more homozygous integration sites, the positive ratio will be 100%; if there are two or more separate hemizygous integration sites, the positive ratio will be $1-(1/2)^n$ (n indicates the number of integration sites). Since the positive ratios were 96% and 94%, the number of integration sites could be deduced as 4-5, which is accordant to the goodness-of-fit test ($P<0.05$).

Plasmid rescue

Several hundred colonies containing DNA from fish A and B were picked up from *Bam*HI digested genomic DNA, among which two hundred clones for each fish were analyzed by *Eco*RI and *Bam*HI digestion. The transgenes from fish A were classified into six classes while those from fish B into four classes according to the electrophoretic patterns. The details were shown in Tab. 1.

On the other hand, when *Kpn*I was used for digestion, several hundreds of colonies from two individuals were grown on LB plates containing ampicillin. 30 clones were randomly picked up from the plates and analyzed by multiple-restriction-enzyme digestion, which revealed that 73% (22/30) of the recovered transgenes were the same as the plasmid pMThGH on molecular configuration. This result gave another evidence that the transgenes were arranged as head-to-tail arrays in the host genome, since only tandemly arrayed transgenes could be recovered by *Kpn*I digestion (see Fig. 1B).

Restriction maps of recovered transgenes

The restriction maps for the ten classes of the recovered transgenes were made as described in Fig. 4. According to the restriction maps, classes A1 and B1 were the same as pMThGH, which was used for producing the transgenic founders, while the other types were different from pMThGH on both molecular weight and composition. Between the two series, the classes B2, B3 and B4 are the same as A2, A4 and A5, respectively. The variation of transgenes in host fish suggested that parts of the sequences in pMThGH transgene had been rearranged or deleted during the course of integration and four-generation transmission.

***MThGH* fragment detected by PCR analysis and Southern blot**

All the classes of the recovered transgenes could be hybridized against *MThGH* probe (Fig. 5A, B), indicating that all the recovered transgenes maintained complete or partial *MThGH* sequence. PCR analysis showed that A1, A2, A3, A4, A5, B1, B3 and B4 gave specific amplification band identical to pMThGH. However, A6 and B2 did not produce any specific amplification (Fig. 5C). This suggested that deletion or mutation must have occurred in the PCR primer binding sequences in those transgenes. We
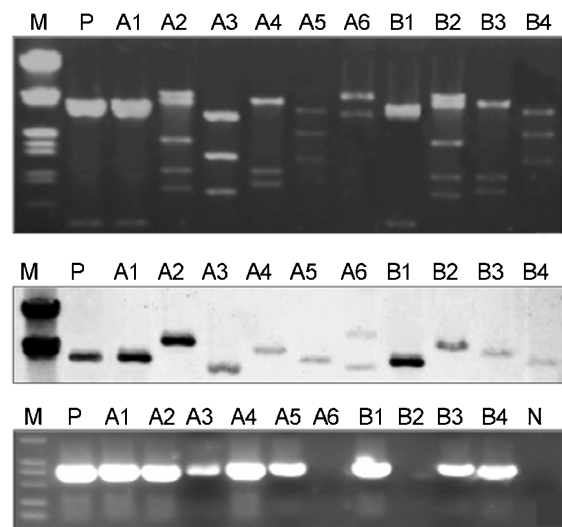

Fig. 5 Location and existence of the *MThGH*-fragment in recovered transgene clones of F4 transgenic fish. (**A**) Analysis of enzyme digestion of transgene clones. M, λ DNA/*Eco*RI+*Hind*III marker; P, pMThGH was digested with *Bam*HI and *Eco*RI; A1, A2, B1 and B2 were digested with *Bam*HI and *Eco*RI, A3 was digested with *Pst*I, A4 and B3 were digested with *Bam*HI, A5 and B4 was digested with *Hind*III and *Bgl*II, A6 was digested with *Hind*III and *Eco*RI. (**B**) Southern blot analysis after enzyme-digestion. Probes are *MThGH* and l DNA marker labeled with DIG. M, λ DNA/*Eco*RI+*Hind*III marker; pMThGH and transgene clones were digested as described above. (**C**) PCR analysis of recovered clones with P+ and P-. M, DL2000 marker; P, positive control; N, negative control.

noticed that although the restriction map of B2 was the same as A2, PCR analysis showed different results, indicating that primer binding sequences in B2 having changed a lot in base composition but not in length.

Host DNA fragment adjacent to the transgenes

Three types of host DNA fragments adjacent to the transgenes in both fish A and B were subcloned into pUC19 and analyzed. Firstly, transgene fragments in the recovered clones were examined by Southern hybridization using DIG-labeled *MThGH* probe (Fig. 5). In A1 and B1, there was a 3.5 kb *Eco*RI hybridization fragment, i.e. the transgene *MThGH* retains its integrity. In the aberrant classes, hybridization signals appeared at a 5.4 kb *Bam*HI–*Eco*RI fragment in A2 and B2, a 3.4 kb *Pst*I fragment in A3, a 4.7 kb *Bam*HI fragment in A4 and B3, and a 3.6 kb *Hind*III–*Bgl*II fragment in A5 and B4, respectively. In A6, both 5.2 kb and 3.2 kb *Hind*III–*Eco*RI fragments were detected using the *MThGH* probe. We refer to these hybridized fragments as "hot fragments".

In A2, a 3.2 kb *Hind*III fragment next to the "hot frag-

ment" was subcloned and sequenced from both ends. A 567 bp fragment from one end was sequenced in which NT 116–481 was homologous to 5' regulatory sequence of the *mouse phosphoglycerate kinase-1* gene (gi|200323-|gb|M18735.1|) with 99% identity (Genebank, accession number AF353996). A 547 bp fragment from the other end was sequenced, whose NT 145–313 and NT 334–458 were homologous to the 3' downstream regulation region of *mouse phosphoglycerate kinase-1b*, including the polyA signal (gi|53670|emb|X15340.1|) with 99% and 97% identity, respectively (Genebank, accession number AF353995). This implied that the 3.2 kb *Hind*III fragment of A2 was the phosphoglycerate kinase-1 homologue of common carp.

In A3, a 1.5 kb *Pst*I fragment next to the 3.4 kb *Pst*I "hot fragment" was analyzed. A 609 bp DNA fragment from one end was found 98% homologous to the upstream of *human epidermal keratin 14 (KRT14)* gene promoter region (gi|533529|gb|U11076.1|HSU11076) (Genebank, accession number AF353994). The sequence from the other end could not be identified because of GC rich clusters.

In A6, a 1.0 kb *Hind*III–*Bam*HI fragment at one end of "hot fragment" was analyzed. A 698 bp DNA sequence from *Hind*III→*Bam*HI direction was homologous to the promoter and 5'UTR region of *common carp β-actin* gene (gi|5881101|gb|AF170915.1|AF170915) with 100% and 99% identity at NT 1–230 and NT 241–686, respectively. A 1.1 kb *Bgl*II–*Bam*HI fragment at the other end of "hot fragment" was also analyzed. It was found that a 694 bp DNA sequence at *Bgl*II→B*am*HI direction was 99% homologous to *common carp β-actin* gene intron A (gi|21-3041|gb|M24113.1|). It is obvious that the sequence adjacent to the transgene of A6 was *common carp β-actin* gene.

Furthermore, with each set of primers designed according to the fragment sequences, PCR analysis gave unique and distinct amplification among the F4 transgenic fish and the non-transgenic fish (data not shown), indicating that the recovered adjacent sequences did belong to the genome of common carp.

DISCUSSION

The present investigation provides new information about transgenes in transgenic common carp through four generations of transmission.

Germline transmission of transgene to F4 offspring

We previously found that the integration of foreign genes occurred during the embryogenesis, resulting in random multiple-site integration of transgene and transgenic mosaicism [4]. Since the F4 transgenic fish were raised from one transgenic male and four transgenic females, F4

transgenics would inevitably contain various integration patterns. In this study, we found that each F4 transgenic genome contained about 200 copies of transgene, which were concatameric arrays at 4-5 insertion sites. It was also found that the transgene concatemers existed as head-to-tail arrays, which is quite consistent to the order of repetitive DNA in normal genome. This implies that the transgene concatemers were formed with a mechanism similar to the formation of repetitive DNA in animal genome. Since the transgene concatemers were presumably formed in the egg or early embryo of injected founders [4], it is reasonable to assume that the transgene concatemers in F4 genome were transmitted from the transgenic founders *via* germline.

Among all the recovered transgenes, although they were characterized into 6 classes, more than 83% maintained their original configuration. Since the transgenes were recovered through *Bam*HI digestion, self-ligation, transformation and ampicillin selection, it could be concluded that these transgenes in F4 genome maintained the *Bam*HI cohesive ends, the plasmid replicon and *Amp^r* sequences. In addition, most of the recovered transgenes were the same as the injected plasmid in both molecular weight and multiple-restriction-enzyme recognition. These results revealed that most transgenes were inherited faithfully through four-generation transmission. As a result of the faithful germline transmission of pMThGH transgene, F4 transgenic common carp would as expected have inherited the genetic traits such as growth enhancement [10].

Polymorphism of transgene in F4 genome

According to restriction maps, except A1 and B1 (with original configuration), other classes (less than 17% in proportion) were distinctly different from the original configuration of pMThGH in molecular weight and restriction enzyme recognition, suggesting that parts of the sequences in aberrant transgenes had been rearranged or deleted during the course of integration and inheritance. Although B2 was the same as A2 based on restriction analysis, different results were obtained from PCR analysis, suggesting that mutations or rearrangements occurred and there were some changes which could not be detected by restriction analysis. In addition, all the four classes of transgenes recovered from fish B could be found in fish A and the proportion of each among the four classes was similar between the two F4 individuals. This could be explained by that the polymorphic integration patterns of transgenes in these two F4 individuals were derived from the transgenic parents.

According to the present study, the possible reasons for polymorphism of transgene integration in F4 genome could be speculated as following: (1) Independent assort-

ment of chromorsomes during four-generation transmission. Since the transgenic founders received randomly and mosaically integrated transgenes as described by our previous studies [4], the transgenic offspring derived from hybridization between transgenic individuals will contain various integration patterns. In previous studies, e.g., it was found that two or three integration sites existed in the genome of P0 transgenic common carp [23] and widespread mosaicism existed in transgenic founders of rainbow trout (*Oncorhynchus mykiss*) [24]. (2) Recombination during the course of integration and germline transmission. Recombination is thought to be very important for the integration of transgene [25, 26]. Massive rearrangement of genomic DNA including deletion or translocation was observed at the integration site and the flanking region of the transgene in transgenic rice and transgenic rat [25, 27]. The present study showed that 5 classes among 6 classes of the transgenes underwent great changes in restriction maps, implying that recombination occurs between transgene and transgene, or between transgene and host genome. Moreover, since there were several kinds of transgene concatemers in the transgenic founders, this might allow the generation of alleles with different repeat numbers due to misalignment during genetic recombination. (3) Mutation or rearrangement of transgene during germline transmission. In our study, although it was found that the class B2 was the same as A2 on both molecular weight and restriction map, mutations or rearrangement occurred and resulted in different results of PCR products.

Analysis of integration sites

Multiple integration sites of transgene existed in the F4 genome, suggesting that the integration of transgene occurred randomly in the transgenic founders. For example, transgene of class A2 was integrated into the common carp sequence homologous to the *mouse phosphoglycerate kinase-1* gene, that of class A3 was flanked by common carp sequence homologous to *human epidermal keratin 14 (KRT14)* gene and that of A6 was integrated into common carp *β-actin* gene. It is surprising that although these transgenes were integrated into coding or regulatory sequences of some housekeeping genes, the resulted fish did not suffer from any insertional mutagenesis. It may be due to that those transgenic fish carry inserted transgenes in a hemizygous status. In previous studies, however, the existence of integration hotspots had been proposed in some cases. DNA topoisomerase I or II binding sites were found to cluster around the junctions; short, purine-rich tracts, some short forward and reverse overlapping sequences or short, direct repeats consisting of 4-6 bp, AT-rich S/MAR were present, either at the junction site or in the immediate flanking regions [15, 26–30]. In the present

study, similar results were found. Among the identified three sequences adjacent to the transgenes of F4 transgenic fish, a 188 bp tract in A3 (NT 313–501) was found 94% homologous to the "MIR" repeat family (gi|10280853: c51526–51336), and it may be involved in the course of transgene integration.

Due to the limitation of the nature of plasmid rescue, we could not recover those transgenes which had lost the plasmid replicon or ampcilin resistance region. This resulted in the underestimation of transgene classes. However, the present study demonstrated a relatively comprehensive situation of the transgene integration pattern in F4 transgenic fish.

ACKNOWLEDGEMENTS

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