Isolation and Linkage Mapping of Coding Sequences from Chicken Cosmids by Exon Trapping

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ABSTRACT : We performed exon trapping in order to locate functional genes on chicken chromosomes (GGA) and to identify functional gene sequences from chicken cosmids. Sequence analysis of 100 clones revealed 17 putative exons, five of which were identified with known sequences in a gene database search: thymopoietin beta (*TMPO*), U5 snRNP-specific 40 kDa protein (*HPRP8BP*), dihydropyridine receptor alpha 1 subunit (*CACNL1A3*), cystein string protein (*CPS*) and *C15orf4*. We attempted to map the genes to chicken chromosomes by using FISH and linkage analysis. The chromosomal localizations were GGA1 (*TMPO*), GGA10 (*C15orf4*), GGA23 (*HPRP8BP*) and GGA28 (*CPS*) by FISH and linkage analysis, while that of *CACNL1A3* was predicted to be on a microchromosome by FISH but not by linkage analysis. Comparative mapping analyses between chickens and humans for the genes revealed both known and new synteny. The syntenic conservation between GGA1 and human chromosome (HSA) 12q23 (*TMPO*) and between GGA10 and HSA15q25 (*C15orf4*), were consistent with a recent publication, while two new syntenies were observed between GGA28 and HSA20q13.3 in *CPS* and between GGA23 and HSA1p34-35 in *HPRP8BP*. The information of presently mapped genes can contribute as anchor markers based on functional genes and the construction of a comparative map. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 3 : 309-312)***

Key Words : Exon Trapping, Chicken, Linkage Mapping, FISH, Comparative Mapping

INTRODUCTION

Exon trapping is a rapid and efficient technique for identifying expressed gene sequences, based on the selection of functionally splice sites in genomic DNA (Church et al., 1994). Although there are other current methods for identifying genes, exon trapping has an advantage in that it does not require any prior knowledge of the tissue-specific transcriptional status and can be easily performed on a complex genome (Comincini et al., 1997).

Chicken linkage maps have been constructed during the past decade (Groenen et al., 2000; Lee et al., 2002). Comparative maps among chickens, humans and mice have also been constructed by using orthologous genes and anonymous loci (Schmid et al., 2000). Subsequently, a global image of the chromosomal construction among them has been obtained. However, a more comprehensive comparative map is now required in order to identify genes responsible for disease (Yoshizawa et al., 2003) and also to investigate chromosomal evolution, especially for microchromosomes. The incrementation of functional genes mapped to the chromosome will contribute to the construction of a high-resolution comparative map.

Our work is the first application of exon trapping in chickens to map functional genes to chicken chromosomes. The functional genes obtained by exon trapping were mapped to chicken chromosomes by FISH and linkage analysis, then the homologous regions between chickens and humans were investigated.

MATERIALS AND METHODS

Exon trapping

Exon trapping was performed using chicken genomic cosmid library (Clontech, CA). The whole procedure is based on the Exon Trapping System (GIBCO BRL, MD). Twenty single DNA pools from each five cosmids (total 100 cosmids) were digested with a combination of *Pst*I and *Pvu*I restriction endonucleases (New England BioLabs, MA), purified and ligated to pSPL3 trapping vector. The ligated products were transformed into HB101 *E. coli* cells. Plasmid DNA was isolated after growing in liquid culture. The recombinant plasmid DNA was transfected into 60% to 80% confluent COS-7 cells (Riken Cell Bank, Tsukuba, Japan) by using Effectene Transfection Reagent (QIAGEN, CA). The cells were incubated for 40 h after the transfection.

RNA was extracted by Sepasol-RNAI (nacalai tesque, Japan) and cDNA synthesis was performed. In order to eliminate vector-only and false positive products, BstXI restriction endonuclease was added directly to the reactions overnight. Secondary PCR amplification was performed by SD2 and SA4 primer (GIBCO BRL, MD). The product was run by 2% agarose gel electrophoresis and then the adequate size of PCR products (160 bp to 450 bp) were recovered from the gel using Mate Gelpure DNA

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Table 1. Sequences of primers for cosmid sequencing and linkage analysis

Primer name	Sequence (5' to 3')
Cosmid vector F	AAAGTGCCACCTGACGTCTAAGAAACC
Cosmid vector R	AGATTTCATACACGGTGCCTGACTGCG
TMPO exon F[a]	AGTGGTTTCTTTGGTGTTCTTCTGGGC
TMPO exon R[a]	CAAGATGGAGTTACTGAGACTGTCTGG
C15orf4 exon F	CGAACATCATTGAACAGAAAGCTGGAC
C15orf4 exon R[a]	GCTCAACTGTCTTCGCAGAGTCTCTCC
C15orf4 genome[a]	TCAGTTGGAGGAAGGAGTAGCAGTAGG
CPS exon F[a]	CACTTGTCTTAGAATAGGCAAACCAGG
CPS exon R	ATTCTAAGACAAGTGGTAGCTGCCTCC
CPS genome[a]	AACATAGGAGCGATTCTACTCAACGGC
HPRP8BP exon F[a]	GAGAGATGTGTAAAGATTTTCCAGGGG
HPRP8BP exon R	AATCTTTACACATCTCTCTTTCGGGGC
HPRP8BP genome[a]	CAGTTCAATTTCCAAAGGAGATTTAGC

[a] These primers were used to amplify for chicken genomic DNA. F and R indicate the exon specific primers in each gene.
TMPO: thymopoietin beta, HPRP8BP: U5 snRNP-specific 40 kDa protein, CACNL1A3: dihydropyridine receptor alpha 1 subunit, CPS: cystein string protein.

purification Kit (ISC BIO EXPRESS, UT). Finally, the products were ligated to the pGEM-T Easy Vector (Promega, WI) and transformed into JM109.

Characterization of trapped exons and sequence analysis

White single colonies were picked up and used for PCR amplification with SD2 and SA4 primers. Positive clones with an insert larger than the 153 bp that derived from self-splicing of the vector, were sequenced by using SequiTherm EXCEL II DNA sequencing kits (Epicentre Technologies, Madison, WI) with dyed vector primers and analyzed on a LI-COR automatic sequencer (model 4200L, LI-COR, NE). Sequences were analyzed by using BLAST search. The sequences of the trapped exons have been deposited at the DDBJ database under the accession numbers AB114863-AB114867.

FISH

FISH was performed basically according to Matsuda and Chapman (1995). Lymphocytes were isolated from the blood of female adult chicken, and transferred to 5 ml A_{MNIO}MAX-C100 medium (GIBCO BRL, MD) with 0.06% glutamine and 50 μg/ml HA15 (Murex). The lymphocytes were cultured for 66 h at 39°C. Colcemid (0.04 μg/ml) was added 2 h before harvesting the cells. The DNA probes were labeled by nick translation kit (Roche, Swiss) and the hybridized probes were stained with avidin-FITC (Roche, Swiss). The slides were stained with 1 μg/ml propidium iodide for observation.

Identification of polymorphisms and linkage analysis

Partial genomic sequences flanking cosmid vector were determined by sequencing with cosmid vector primers. Primers were designed based on the genomic sequences to amplify from the region flanking cosmid vector to the region of trapped exons. Primers within exon sequences were also designed. All primers amplified successfully were listed in Table 1. The PCR reactions were carried out with 1 ng of purified cosmid DNA as a template in a volume of 50 μl of 1×reaction buffer; 400 μM dNTPs; 0.5 μM of each primer; and 2.5 U of LA Taq polymerase (Takara Shuzo Co., Tokyo, Japan). An initial denaturation period of 1 min at 94°C was followed by 30 cycles for 10 s at 98°C, 15 min at 68°C and a final extension period of 10 min at 72°C.

The PCR products were digested with several restriction endonucleases to detect polymorphisms between parents of the Kobe University (KU) resource family. The KU family had been established in previous study (Lee et al., 2002). The resource family consists of 380 backcross chicks produced by crossing a single male of the White leghorn WL-F line to eight F1 females produced by crossing the male and a Fayoumi OPN line female. A subset of 55 backcross chicks from one F1 female was used for linkage analysis in this study. These polymorphisms were analyzed with previously published information of KU linkage map (Lee et al., 2002). Linkage groups were determined by two-point analysis under linkage criterion of $p<0.05$ as a G-statistic for independence and Kosambi function, using Map Manager QTXPb12 (Manly et al., 2001).

RESULTS AND DISCUSSION

In total, 100 clones were analyzed for the presence of an insert by PCR. The products ranged in size from 160 to 500 bp. After a selection based on size, 34 clones were sequenced with the PCR products directly as templates. The lengths of trapped exons were from 24 bp to 327 bp. The DNA sequences were analyzed by using BLAST search (http://www.ncbi.nlm.nih.gov/blast/). Seventeen clones possessed the putative trapped exons contained in sequences derived from the intron of the pSPL3 trapping vector.

Table 2. Results of sequence database searches for the trapped exons

Cosmid	Size (bp)	Gene (locus name)	Sp.	Accession	% Sim
A1	173	EST	CK	BI390001	99
		C15orf4	HU	AF210056	56
F8	68	EST	CK	AJ395662	100
		Cystein string protein (*CPS*)	RA	S81917	87
M1	325	Thymopoietin beta (*TMPO*)	HU	U09087	84
S21	83	U5 snRNP-Specific 40 kDa protein (*HPRP8BP*)	HU	XM_001618	99
J1	106	Dihydropyridine receptor alphs 1 subunit (*CACNL1A3*)	HU	U30667	85

CK: chicken, HU: human, RA: rat, EST: expressed sequence tag.

Table 3. Results of sequence database searches and deduced chromosomal localization of the trapped exons

Gene	Cosmid	Size (kbp)	Polymorphism type	Chromosomal localization		
				Chicken		Human
				FISH	Linkage	
C15orf4	A1	7	PCR-RFLP (*Msp*I)	micro	10	15q25
CPS	F8	1.9	Null allele	micro	28	20q13.3
TMPO	M1	1	PCR-RFLP (*Dra*I)	1p	1	12q23
HPRP8BP	S21	0.5	Insertion/deletion	micro	23	1p34-35
CACNL1A3	J1	ND	No polymorphism	micro	NA	1q31-32

NA: not assigned, ND: not detected. *TMPO*: thymopoietin beta, *HPRP8BP*: U5 snRNP-specific 40 kDa protein, *CACNL1A3*: dihydropyridine receptor alpha 1 subunit, *CPS*: cystein string protein, micro: microchromosome.

Five sequences out of 17 clones were identified as known genes. Table 2 summarizes the results. Three were the genes for human thymopoietin beta (*TMPO*, M1), U5 snRNP-specific 40 kDa protein (*HPRP8BP*, S21) and dihydropyridine receptor alpha 1 subunit (*CACNL1A3*, J1). Two were expressed sequence tags (EST) for chickens (A1, F8); these EST sequences were reanalyzed by BLAST search. The EST AJ395662 was similar to rat cystein string protein (*CPS*) and the EST BI390001 to human *C15orf4*. The trapped sequence of *TMPO* was included in three exons, while the other four gene sequences were consistent with a single exon for each gene. Additionally, 12 clones were not similar to any entry in the database.

The parent cosmids of five clones were identified by using the PCR method with the exon specific primers (Table 1). Five cosmids were used for mapping to chicken chromosomes by FISH. Consequently, one clone was mapped to chicken chromosome (GGA) 1q, while four on chicken microchromosomes could not be identified by chromosomal numbers (Table 3).

We therefore attempted to locate these genes on the chicken chromosome by linkage analysis using the Kobe University (KU) resource family (Lee et al., 2002). Polymorphisms for each gene between parents of the KU family were detected as follows. PCR was performed for parental cosmid DNA with primers of cosmid vector and trapped-exon sequences (Table 1). Four out of five clones (A1, F8, M1 and S21) were successfully amplified, and the lengths were approximately 7 kbp (A1), 1.9 kbp (F8), 1 kbp (M1) and 0.5 kbp (S21). No PCR product was observed for the J1 clone. Genomic sequences flanking the cosmid vector were determined by using the PCR products and then primers for chicken genomic sequences were designed (Table 1). The flanking genomic sequence of *TMPO* revealed a coding sequence, so that the primer was designed within the exon sequence.

Using these primers, we conducted PCR reactions for genomic DNA of the KU family parents as templates. The PCR product of *CPS* revealed null allele in male chickens, and that of *HPRP8BP* exhibited size differences between parents. For *TMPO* and *C15orf4*, several restriction endonucleases were used to detect the polymorphisms. Consequently, *Msp*I digestion yielded polymorphic bands for *TMPO* PCR products, and *Dra*I digestion yielded polymorphic bands for *C15orf4* PCR products. These polymorphisms were analyzed for the KU resource family to locate the genes. The benefit of this strategy is the possibility of a wide screening of spacer or intron regions within cosmid inserts to detect the nucleotide polymorphisms in the reference family.

As a result of linkage analysis, four genes were located on chicken chromosomes (Figure 1 and Table 3). The *TMPO* gene was mapped at 142.2 cM from the distal end of GGA1p. The *C15orf4* was on GGA10 at the 46.4 cM position, HPRP8BP at the terminal end of GGA23 and *CPS* at the terminal end of GGA28q. The human chromosome (HSA) localizations of these genes have been mapped at 12q23 (*TMPO*), 15q25 (*C15orf4*), 1p34-35 (*HPRP8BP*) and 20q13.3 (*CPS*) and *CACNL1A3* was at 1q31-32, although the position of this gene could not be located by the chicken linkage map (Table 3).

The syntenies for *TMPO* (GGA1p vs. HSA12q23) and *C15orf4* (GGA10 vs. 15q25) are consistent with a recent publication of a comparative genome map of humans and chickens (Schmid et al., 2000). However, the comparative

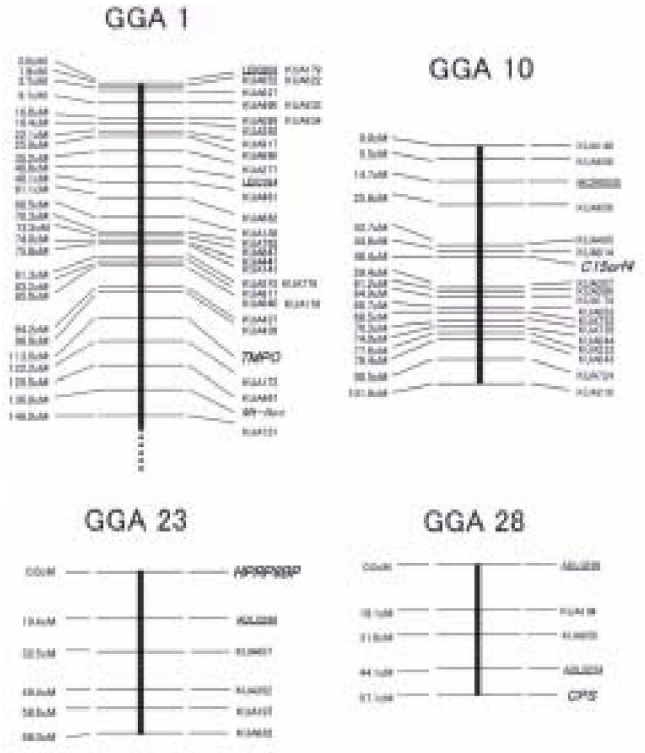

Figure 1. Chromosomal localization of trapped functional genes. Linkage map was produced by using the Kobe University resource family. Functional genes are shown in italic and trapped functional genes in this study are shown in italic and boldface type. Microsatellite markers are underlined. KUA indicates Kobe University AFLP markers (Leeet al., 2002). Distances on the markers are given in cM (Kosambi map function). *TMPO*: thymopoietin beta, *HPRP8BP*: U5 snRNP-specific 40 kDa protein, *CACNL1A3*: dihydropyridine receptor alpha 1 subunit, *CPS*: cystein string protein, *Mt-Aco*: mitochondrial aconitase.

map did not illustrate the synteny between chicken GGA28 and HSA20q shown for *CPS* and there is no functional gene on GGA23 in the map. Consequently, in this study, the synteny between GGA23 and HSA1p34-35 exhibited for *HPRP8BP* is a new finding. Therefore, the chromosomal localizations and the synteny would contribute to a more detailed comparative map between chickens and humans.

In this study, we found that exon trapping is a useful and effective approach for isolating and mapping functional genes in chickens. Additionally, we presented a strategy to locate the exon trapped genes on the linkage map. These techniques will be very useful to develop the functional gene markers located on the chicken linkage map. The approach would be used to identify responsible genes within the candidate region for chicken disease or economic traits (Yonash et al., 1999; Yoshizawa et al., 2003). As a result, the information of presently mapped genes could contribute as anchor markers based on functional genes and to the construction of a comparative map between chickens and other species.

IMPLICATIONS

Full sequencing of the chicken genome is underway by both the whole genome shotgun method and clone-based sequencing. A linkage map is an essential guide for navigation, allowing the location of any gene or other landmark in the chromosomal DNA. Exon trapping is a valuable tool for identifying expressed gene sequences. Functional genes that are mapped using this strategy would contribute as anchor markers and to the construction of a comparative map between chickens and other species.

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