Cloning and Characterization of Liver cDNAs That Are Differentially Expressed between Chicken Hybrids and Their Parents

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ABSTRACT : Using mRNA differential display technique, we investigated differential gene expression in hybrids relative to their parents in a diallel cross involving four chicken breeds in order to provide an insight into the molecular basis of heterosis in chicken. The results indicated that there was extensive differential gene expression between chicken F1 hybrids and their parents which was classified into four kinds of patterns as following: (1) bands only detected in hybrid F1; (2) bands only absent in hybrid F1; (3) bands only detected in parent P1 or P2; (4) bands absent in parent P1 or P2. Forty-two differentially expressed cDNAs were cloned and sequenced, and their expression patterns were confirmed by Reverse-Northern dot blot. Sequence analysis and database searches revealed that genes showed differential expression between hybrid and parents were regulatory and functional genes involved in metabolism, mRNA splicing, transcriptional regulation, cell cycles and protein modification. These results indicated that hybridization between two parents can cause changes in expression of a variety of genes. In conclusion, that the altered pattern of gene expression in hybrids may be responsible for heterosis in chickens. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 12 : 1684-1690)***

Key Words : mRNA Differential Display, Differential Gene Expression, Heterosis, Chicken

INTRODUCTION

Heterosis or hybrid vigor has been recognized for almost a century and exploited extensively in both animal and plant breeding all over the world (Duvick, 2001; Wu, et al., 2002), however, up to now, its genetic background remains unknown. Three, namely, dominance (Davenport, 1908; Bruce, 1910), over-dominance (East, 1908; Shull, 1908) and epistasis (Templeton, 1979, 1980; Allward, 1996) hypotheses, were proposed to explain the genetic basis of the phenomenon. Based on QTL mapping, recent studies conducted by Stuber et al. (1992), Xiao et al. (1995) and Yu et al. (1997) on maize and rice heterosis have supported the hypotheses of overdominance, dominance and epistasis, respectively.

The relationships between gene expression patterns and heterosis were also studied. Although the genome in hybrid F1 is derived its parents, hybrid performance is often quite different from either of the parents, implying that differences in gene expression between hybrids and their parents are responsible for heterosis (Sun et al., 1999). Romagnoli et al. (1990) first found that patterns of gene expression in terms of mRNA and protein quantities were altered in a heterotic maize hybrid as compared to its parental inbreds, then Tsaftaris et al. (1995, 1998) also reported that the mean mRNA content for 35 tested gene loci was higher in the highly heterotic hybrid than in the low heterotic hybrid and their parental inbred lines. Recently, by using DDRT-PCR, it was reported that there were significant gene differential expression between F1 hybrids and their parents in rice (Cheng et al., 1996, 1997; Xiong et al., 1998), wheat (Sun et al., 1999; Xie et al., 2003), maize (Tian et al., 2002, 2003), chicken (Wang et al., 2004; 2005) and pig (Liu et al., 2004). By for, few reports on molecular genetic basis of domestic animal heterosis in terms of differential gene expression between hybrids and their parents have been found, because it is more difficult to gain absolute purebred or pure line and enough large samples than plants (Wang et al., 2004, 2005; Yu et al., 2004; Li et al., 2005). Only Wang et al. (2004, 2005) have reported there was differential gene expression between chicken F1 hybrids and their parents and its relationship to heterosis of meat traits.

In the present study, we conducted a differential display analysis in liver tissue between twelve 8-week-old chicken F1 hybrids and their parents. In order to further analyze the relationship of the differentially displayed genes to heterosis, Forty-two cDNAs derived from genes that are differentially expressed were cloned and sequenced, and their possible roles in heterosis were discussed.

MATERIALS AND METHODS

Animals

4×4 diallel crossing were performed involving four chicken breeds, which were White Leghorn (A), Silkies (C), CAU Brown (D) and White Plymouth Rock (E), sixteen crosses including a total of 3,200 individuals were gained, namely, AA, CC, DD, EE, AC, CA, AD, DA, AE, EA, CE, EC, CD, DC, DE and ED, 100 males and 100 females being

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Table 1. Heterosis percentage (%) of nine carcass traits from hybrids of 8-week-old chicken

traits / Hybrids	BMY	LMY	WW	EWG	EW	BL	SL	AFW	IFW
AC	5.01	7.24	6.71	6.96	6.57	-0.61	2.83	20.45	6.78
AD	5.57	5.89	6.71	5.73	7.05	3.10	2.96	23.92	14.04
AE	-17.79	-18.99	-12.27	-15.27	-16.56	2.02	3.93	3.64	-2.89
CA	10.46	14.88	5.35	9.50	9.87	3.88	1.70	6.07	23.73
CD	18.62	22.07	8.85	6.63	13.96	7.16	5.41	70.53	21.31
CE	-24.54	-18.81	-13.29	-17.12	-19.82	5.17	5.00	15.46	5.56
DA	-1.76	-2.61	-8.66	-3.84	-2.95	0.60	0.77	-22.99	12.28
DC	5.55	5.28	-1.04	2.92	2.55	3.96	2.42	41.41	8.20
DE	-20.26	-12.24	-7.84	-14.48	-12.76	2.01	2.50	0.75	-7.55
EA	-24.03	-20.21	-13.56	-18.29	-20.51	1.35	-2.71	-36.60	-10.58
EC	-32.49	-20.50	-15.59	-19.21	-23.19	1.63	6.93	-9.49	9.26
ED	-25.67	-15.64	-9.27	-13.84	-16.55	2.12	8.28	-17.29	4.72

BMY: Breast muscle yield; LMY: Leg muscle yield; WW: Wing weight; EWG: Eviscerated weight with giblet.
EW: Eviscerated weight; BL: Body length; SL: Shankbone length; AFW: Abdomen fat weight; IFW: Iintermuscular fat width.

included per cross. Thirty male individuals of each cross were randomly selected and measured for nine carcass traits at 8 weeks old and heterosis percentages were calculated (Table 1), which results showed there was significant heterosis for every trait in each hybrid cross. For ensuring that traits of hybrids and their parents were measured under the same condition to eliminate the systematic environmental error, phenotypic values for nine traits of four parents (A, C, D, E) were represented by those of four pure breeding crosses (AA, CC, DD, EE).

The fresh liver tissues of 8 healthy males were randomly collected from twelve F1 hybrids and four pure breeding crosses respectively at 8 weeks old for mRNA differential display analysis, and then stored at liquid nitrogen.

RNA extraction

Total RNA was isolated from each sample using the TRIZOL reagent methods (Gibco-BRL). The minor DNA among the total RNA was digested using DNase (Promega) (Liang and Pardee, 1992). The total RNAs of 8 individuals from every cross was pooled and adjusted to a final concentration of 0.24 μg/μl.

Reverse transcription

Reverse transcription was used to synthesize the first cDNA strand. In a 20 μl reaction mixture containing 0.48 μg of total RNA, 50 mM Tris-HCL (pH 8.3), 40 mM KCL, 7 mM $MgCl_2$, 10 mM DTT, 20 mM dNTP, 200 pM of 3' end anchored primer (HT11G: 5'-AAGCTTTTTTTTTTG -3'). The mixture was incubated at 65°C for 5 min, 37°C for 10 min, then 100 U of M-MLV reverse transcriptase (Promega) was added, 37°C for 1 h continued, 75°C for 5 min.

PCR amplification of cDNA

The following primers were synthesized according to von der Kammer et al. (1999). As 3' end anchored primers, the oligonucleotides H-T11G (5′-AAGCTTTTTTTTTTG-3′), H-T11C (5′-AAGCTTTTTTTTTTC-3′) and H-T11A (5′-AAGCTTTTTTTTTTA-3′) were used. DD6 (5′-TGC CGAAGCTTTGGTACC-3′), DD10 (5′-TGCCGAAGCTT TGGTAGC-3′), DD15 (5′-TGCCGAAGCTTTGGTATG-3′), DD18 (5′-TGCCGAAGCTTTGGTCAC-3′), DD19 (5′-TG CCGAAGCTTTGGTCAG-3′), DD20 (5′-TGCCGAAGCT TTGGTCAT-3′), DD34 (5′-TGCCGAAGCTTTGGTGAC-3′) and DD46 (5′-TGCCGAAGCTTTGGTGTC-3′) served as 5′end primers. The template cDNA was amplified in a total volume of 20 μl by mRNA differential display method. The PCR mixture contained 2 μl of reverse transcription product, 10 mM Tris-HCL (pH 9.0), 50 mM KCL, 1.5 mM $MgCl_2$, 20 mM dNTPs, 200 pM primers and 1 U of Taq DNA polymerase (Promega). The cycling reaction were predenatured at 94°C for 5 min, followed by 25 cycles of 30 s at 94°C, 60 s at 40°C, 90 s at 72°C, followed by 15 cycles of 30 s at 94°C, 60 s at 52°C, 90 s at 72°C. The final cycle was followed by an extension step at 72°C for 7 min.

Electrophoresis

The PCR amplified products were separated on 4% denaturing polyacrylamide gels (0.4 mm thick) in a temperature-regulated Bio-Rad Sequencing System (Bio-Rad. Fullerton, Calif.), at 50°C. Gels were stained with silver, and photographed.

Reverse-Northern dot blot analysis, cloning and sequencing of differentially expressed cDNA

Bands that showed qualitative differences between hybrid F1 and their parents were excised from the gel and reamplified using the same PCR amplified condition as above. Reverse-Northern dot blot analysis was performed according to the manufacturer's instructions of DIG-HIGH labeling kit (Roche), reverse transcripts products of pooled total RNA of each cross were labeled and hybridized to the

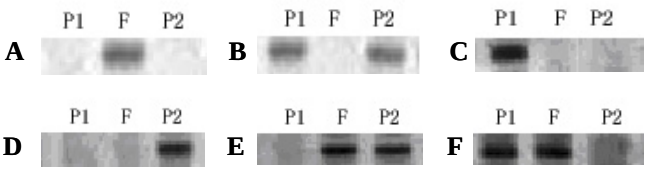

Figure 1. Patterns of differential gene expression between chicken hybrids and their parents. P1: female parent; F: hybrid; P2: male parent. A: Bands detected only in the F_1 but not in either of the parents (UNF_1); B: Bands observed in both parents but not in the F_1 (ABF_1); C: Bands occurring only in female parent but not in F_1 and male (UNP_1); D: Bands occurring only in male parent but not in F_1 and female (UNP_2); E: Bands present in F_1 and male parent but absent in the female (ABP_1); F: Bands present in F_1 and female parent but absent in the male (ABP_2).

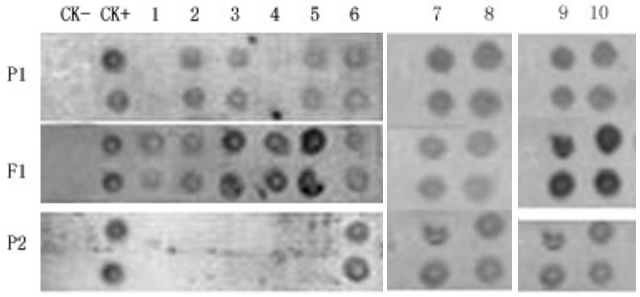

Figure 2. Partial results of reverse-Northern dot blot analysis. CK-: Negative control; CK+: Positive control; Lane 1, 4: UNF1, Bands detected only in the F_1 but not in either of the parents; Lane 2, 3: ABP1, Bands present in F_1 and female parent but absent in the male (ABP_1); Lane 5: HSF, bands observed in both parents and in the hybrid F1 and levels of expression in the hybrid are similar to that in one parent. Lane 7, 8: HL, bands observed in both parents and in the hybrid F1 and levels of expression in the hybrid are lower than that in either of the parents; Lane 9, 10: HH, bands observed in both parents and in the hybrid F1 and level of expression in the hybrid is higher than that in either of the parents.

excised differentially expressed cDNAs respectively. The differentially expressed cDNAs, which were confirmed to be positive by Reverse-Northern dot blot analysis, were ligated into pMD18-T vector (Takara) and transformed into DH5α *E. coli* cells, then they were sequenced (377 DNA sequencer) using universal M13 primer.

Blast analysis

Blast search was performed online using blastn software at the website http://www.ncbi.nlm.nih.gov. All the sequences of differentially expressed cDNAs were blasted to EST and nr databases. Sequences with identities ≥79% or ≤ e-30 within 180 bp region were considered.

RESULTS

Patterns of differentially expressed fragments

To decreasing the number of false positive cDNA fragments, the PCR amplification was repeated twice and only fragments between 200 bp and 1,000 bp which can be repeatedly detected in two reactions were recorded. A total of 5,594 cDNA fragments were amplified from the liver tissues of 8-week-old chicken in sixteen crosses by using 24 primer combinations comprising one of three one-base anchored primers and each of eight different 5' end oligonucleotide primers. For each cross in the diallel set, the patterns of the differentially displayed cDNA bands among the two parents (P_1 and P_2) and the F_1 fell into four categories (Figure 1): (1) bands observed in both parents but not in the F_1 (ABF_1); (2) bands present in F_1 and one parent but absent in the other parent (ABP_1, ABP_2); (3) bands detected only in the F_1 but not in either of the parents (UNF_1); (4) bands occurring only in one parent but not in F_1 and the other parent (UNP_1, UNP_2).

Table 2 presented the number and percentage of differentially displayed bands among the various crosses belonging to each of the four different categories. Obviously, on average, the UNF1 pattern is the least class of differentially displayed cDNA fragments, The ABP1 and ABP2 patterns accounted for the major portion of the differential displayed fragments. It is also clear from Table 2 that there was considerable variation in the four categories among the twelve crosses, indicating that the differences among hybrids may lead to different degree of heterosis among different hybrids.

Cloning, confirmation and sequencing of differentially expressed cDNA

Forty-two cDNA fragments that showed differential expression between the hybrids and their parents were cloned and sequenced. The expression patterns were confirmed by reverse-Northern dot blot analysis, partial hybridization results were seen in Figure 2 and Table 3. Among the 42 differentially expressed cDNA fragments, six showed enhanced expression in the F1 (HH); eleven were only expressed in F1, but absent in both parents (UNF1); one showed weakened expression in the F1 (HL); sixteen were present in one parent and F1, but absent in the other parent (ABP1, ABP2); eight were expressed in F1 and both

Table 2. Percentage of differentially expressed cDNA fragments of each pattern in twelve hybrids (%)

Hybrids	AE	EA	EC	CE	CD	DC	DE	ED	DA	AD	AC	CA	Mean
UNF_1	2.7	4.2	4.3	4.5	1.9	1.5	4.8	6.6	1.1	1.9	1.7	1.5	3.1
ABF_1	9.0	5.5	5.9	4.5	7.0	12.3	15.9	3.4	5.2	7.5	12.6	16.7	8.8
ABP1, ABP2	12.4	15.1	16.6	16.8	11.9	7.0	9.3	18.4	13.0	12.8	7.0	6.7	12.2
UNP1, UNP2	14.9	12.2	8.7	8.5	7.2	12.1	16.4	7.3	8.6	8.8	8.7	8.9	10.2

Table 3. Expression patterns and homologies of differentially expressed fragments

Differentially expressed cDNAs[a]	Patterns based on DDRT	Patterns based on reverse-Northern dot blot[b]	Homologous gene	Accession No.	Identity
1. DDEE1	ABP2	HSF	Chicken EST	BU424378	96%
2. DDEE2	ABP2	HSF	None		
3. DDEE3	ABP2	HSF	None		
4. DDEE4	UNF1	HSF	None		
5. DDEE5	UNF1	UNF1	Chicken Preproalbumin	X60688	97%
6. DDEE6	ABP2	ABP2	Chicken Preproalbumin	X60688	98%
7. DDEE7	UNF1	UNF1	None		
8. DDEE8	UNF1	UNF1	Human Proteosome subunit p112	D44466.1	83%
9. DDEE9	ABP2	HSF	Human embryo lung	BP490924	98%
10. DDEE10	ABP2	HSF	Human splicing factor 3b1 subunit submit	CB018432	94%
11. DDEE11	ABP2	ABP2	None		
12. DDEE12	UNF1	UNF1	None		
13. DDEE13	UNF1	UNF1	None		
14. DDEE14	ABP1	HH	Human Proteosome subunit p112	D44466.1	79%
15. DDEE15	ABP2	ABP2	None		
16. DDEE16	UNF1	UNF1	None		
17. DDEE17	ABP2	ABP2	None		
18. DDEE18	ABP2	ABP2	None		
19. DDEE19	ABP1	HH	Chicken FTF/LRH-1	AB002403.1	98%
20. DDEE20	ABP2	ABP2	None		
21. DDAA1	ABP2	ABP2	Human KIAA1272 protein	XM_046600.6	82%
22. DDAA2	ABP2	HSF	Human Proteosome subunit p112	D44466.1	82%
23. AAEE1	ABF1	HL	None		
24. EECC1	UNF1	UNF1	None		
25. EECC2	ABP1	ABP1	None		
26. EECC3	ABP2	ABP2	None		
27. EECC4	ABP2	HH	Human Proteosome subunit p112	D44466.1	81%
28. EECC5	UNF1	UNF1	Rat cytosolic NADP- dependent isocitrate dehy- drogenase	AW198493	97%
29. EECC6	ABP1	ABP1	None		
30. EECC7	UNF1	UNF1	Chicken EST	BU390643	97%
31. CCDD1	ABP2	ABP2	None		
32. CCDD2	ABP2	ABP2	None		
33. CCDD3	UNF1	HH	Human protein arginine N- methytransferase3	AW355632	99%
34. CCDD4	UNF1	HH	Human aldehyde dehy- drogenase	BG625522	95%
35. CCDD5	UNF1	UNF1	None		
36. CCDD6	ABP2	ABP2	Human EST	AC091044.9	99%
37. CCDD7	UNF1	UNF1	None		
38. CCDD8	ABP2	ABP2	Human KIAA0676 protein	AB014576.1	83%
39. CCDD9	UNF1	HH	Chicken preproalbumin	X60688	97%
40. AACC1	ABP1	HSF	Chicken preproalbumin	X60688	98%
41. AACC2	ABP1	ABP1	None		
42. AACC3	ABP1	ABP1	None		

[a] DDEE1~20, DDAA1~2, AAEE1, EECC1~7, CCDD1~9 and AACC1~3 represented the differentially expressed cDNAs between F1 hybrids and their parents.

[b] HL: Bands observed in both parents and in the hybrid F1 and level of expression in the hybrid is lower than that in either of the parents. HH: bands observed in both parents and in the hybrid F1 and levels of expression in the hybrid are higher than that in either of the parents. HSF: bands observed in both parents and in the hybrid F1 and levels of expression in the hybrid are similar to that in one parent.

parents, but the level of expression in one parent was lower than that in F1 and the other parent (HSF).

The results of blast search showed that four reverse transcripts displayed similarity to chicken preproalbumin gene and other four showed high homology to human proteosome subunit p112. In addition, eight showed high similarity to human splicing factor 3b submit, human embryo lung, rat cytosolic NADP-dependent isocitrate dehydrogenase, human protein arginine N-methytransferase, human aldehyde dehydrogenase, chicken FTF/LRH-1, Human KIAA0676 and KIAA1272 proteins respectively. Twenty-six cDNA were unknown genes. Differential

expression patterns and blast results of forty-two cDNAs were listed in Table 3.

DISCUSSION

Experimental data presented in this paper demonstrated that there are indeed differential gene expression between chicken hybrids and their parents, which is possibly related to heterosis, the same results were also reported in chicken, pig, rice, wheat and maize (Romagnoli et al., 1990; Tsaftaris et al., 1995, 1998; Cheng et al., 1996, 1997; Xiong et al., 1998; Sun et al., 1999; Tian et al., 2002, 2003; Xie et al., 2003; Liu et al., 2004; Wang et al., 2004, 2005). In addition, some researchers have found that the patterns of gene differential expression were significantly correlated to heterosis of some agronomic traits and animal traits (Xiong et al., 1998; Tian et al., 2003; Xie et al., 2003; Wang et al., 2004). Therefore it is probable that the hybrid's genetic performance is not the simple additive product of genetic materials from both parents, but the result of variations in expression of two sets of genes within the hybrid by means of interactions resulting in the occurrence of heterosis, though a hybrid's all genes come from its both parents and there are no exotic genes in the hybrid in terms of genomic constitutes.

Based on the results of the present study, significant alterations on the patterns of differential gene expression were observed in chicken hybrids. In order to identify the role of these differentially expressed genes on the heterosis, it is necessary to isolate their cDNAs and analyze their functions and their relationships to the heterosis. In this study, we found that genes that are differentially expressed between chicken hybrids and their parents include some genes that are involved in fat metabolism, mRNA splicing, cell cycle, transcriptional regulation and protein modification. This results supported that heterosis is the results of complicated interactions among many regulatory and functional genes, which is determined by the complex genetic mechanisms of quantitative traits. DDEE5, DDEE6, CCDD9, AACC1 show high similarity to mRNA of chicken preproalbumin gene which is the premature of serum albumin, albumin is involved in maintaining osmotic pressure of blood plasma and necessary to the normal metabolism (Watanabe, 1996; Brad, 2003). The sequences of DDEE8, DDEE14, DDAA2 and EECC4 are homologous to that of human proteosome subunit p112, the p112 is the largest subunits of human proteosome and related to DNA damnification, tRNA splicing, cell death, growth and development of body (Boldin et al., 1995; Wigle et al., 1999). DDEE9 showed homology to human embryo lung gene which is involved in transcription regulation (Glass et al., 1997). DDEE10 is homologous to human splicing factor 3b1 subunit which plays important roles in the process of formation of mRNA spliceosome and intron splicing (Triezenberg et al., 1988; Berg, 1989; Busch et al., 1990). EECC5 shows high homology to rat cytosolic NADP-dependent isocitrate dehy-drogenase gene (*CIDH*), so it was inferred that EECC5 is the partial fragment of chicken *CIDH* gene, CIDH is a key enzyme catalyzing NADPH formation and biosynthesis of fatty acid (Winkler, 1986; Belfiore at al., 1995), which is associated with fat traits of chicken. The product of DDEE19 is high homologous to chicken Ftz-F1, chicken Ftz-F1 is a member of steroid hormone gene family, which is a transcript factor specifically expressed in sex and adrenal glands and can regulate genes controlling development and function of these tissues (Nomura, 1998; Oba et al., 2000). DDAA1 and CCDD8 is homologous to human KIAA1272 protein gene and human KIAA 0676 protein gene respectively, which functions are unknown. The predicted products of CCDD3 showed homology to human protein arginine N-methytransferase3, this enzyme can make arginine methylation in arginine-enriched protein functional domains which can decrease the interaction between protein and RNA and impact RNA splicing and transportation, so that it maybe indirectly regulate formation of traits (Kim, 1998). The predicted products of CCDD4 is homologous to human aldehyde dehy-drogenase, which catalyze poisonous cymene oxalic aldehyde to pyruvic acid, thereby helping to provide a normal inner condition for development and growth of body (Thornalley, 1996).

In conclusion, results of the present study together with other studies which indicated there are significant correlations between patterns of gene differential expression and heterosis (Xiong et al., 1998; Tian et al., 2003; Xie et al., 2003; Liu et al., 2004; Wang et al., 2004, 2005). The result further supported that the differentially expressed genes between hybrids and their parents may have resulted in the heterosis of quantitative traits by different patterns, some were functional genes which can be the major genes controlling traits or interact with the major genes, some were regulatory genes which can regulate the transcript levels of functional genes and indirectly impact traits. For example, the F1-specific bands (UNF1) might be the genes which can up-regulate the expression of other functional genes, maybe, the specific expression of them in F1 increased the expression of one major functional gene or more genes which might directly or indirectly control the tested trait, so that F1 performs heterosis. On the contrary, bands absent in F_1 and observed in both parent (ABF_1) might be down regulated genes. Bands present in F_1 and one parent but absent in the other parent (ABP_1 and ABP_2) might be related to the classic dominant hypothesis.

In this study, only liver tissue at 8 week-old chicken was used for DDRT-PCR analysis, it could be expected that there might be more differentially expressed genes in different developmental stages and tissue. In order to better identify the role of these differentially expressed genes on

the heterosis, we will isolate the full-length cDNAs and genomic sequences of these genes, then analyze the relationships of their polymorphisms, quantitative mRNA expression and functions to heterosis in foreseen experiments.

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