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Duplicated Spot 14 genes in the chicken: characterization and identification of polymorphisms associated with abdominal fat traits

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Abstract

In mammals, thyroid hormone responsive Spot 14 (THRSP) is a small acidic protein that is predominately expressed in lipogenic tissue (i.e., liver, abdominal fat and the mammary gland). This gene has been postulated to play a role in lipogenesis, since it responds to thyroid hormone stimulation, high glucose levels and it is localized to a chromosomal region implicated in obesity. In this paper, we report the identification and characterization of duplicated polymorphic paralogs of Spot 14 in the chicken, *THRSPα* and *THRSPβ*. Despite low similarity in amino acid (aa) sequence between chickens and mammals, other properties of Spot 14 (i.e., pI, subcellular localization, transcriptional control and functional domains) appear to be highly conserved. Furthermore, a synteny group of *THRSP* and its flanking genes [NADH dehydrogenase (*NDUFC2*) and glucosyltransferase (*ALG8*)] appears to be conserved among chickens, humans, mice and rats. Polymorphic alleles, involving a variable number of tandem repeats (VNTR), were discovered in the putative protein coding region of the duplicated chicken *THRSPα* (9 bp) and *THRSPβ* (6 or 12 bp) genes. Our study shows that the *THRSPα* locus is associated with abdominal fat traits in a broiler × Leghorn resource population.

Keywords: Spot 14 paralogs; VNTR; Transcriptional regulation; Lipogenesis; Synteny group

1. Introduction

The Spot 14 gene, also referred to as thyroid hormone responsive Spot 14 (*THRSP*), encodes a small acidic protein that was discovered in earlier studies of thyroid hormone action in hepatocytes (Seelig et al., 1981; Jump et al., 1984; Liaw and Towle, 1984). Although the exact molecular mechanism is not clear, *THRSP* is implicated as a transcription factor involved in control of lipogenic enzymes. For instance, *THRSP* is only expressed in lipogenic tissue such as liver, fat and the mammary gland (Liaw and Towle, 1984; Jump and Oppenheimer, 1985). *THRSP* mRNA levels are greatly increased by carbohydrate feeding or insulin-injection and decreased by high plasma glucagon levels or by feeding a diet rich in polyunsaturated fatty acids (Jump et al., 1993). Hepatocytes transfected with a *THRSP* antisense oligonucleotide express decreased mRNA levels in enzymes involved in the lipogenic pathway [i.e., ATP-citrate lyase (*ACLY*), fatty acid synthase (*FAS*) and malic enzyme (*ME*)] (Kinlaw et al., 1995; Brown et al., 1997). Although an

Abbreviations: THRSP, thyroid hormone-responsive Spot 14 protein; aa, amino acid; bp, base pair; *NDUFC2*, NADH dehydrogenase; *ALG8*, glucosyltransferase; *ACLY*, ATP-citrate lyase; *FAS*, fatty acid synthase; *ME*, malic enzyme; COUP-TF1, chicken ovalbumin upstream promoter-transcription factor 1; *L-PK*, L-type pyruvate kinase; Sp1, specificity protein 1; TRE, thyroid response elements; FUR, far upstream region; T_3, triiodothyronine; QTL, quantitative trait loci; CAP3, contig assembly program 3; UD, University of Delaware; CR1, chicken repeat 1; EST, expressed sequence tag; SSC, sodium chloride, sodium citrate; BAC, bacterial artificial chromosome; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; IGCRP, Iowa Growth and Composition Resources Population; BBSRC, British Biotechnology and Biological Sciences Research Council; UTR, untranslated region; VNTR, variable number of tandem repeats; kDa, kilo Dalton; pI, isoelectric point; G12, gastrulation-specific protein; SRE, sterol response element; SREBP1c, sterol response element binding protein 1c; ChoRE, carbohydrate response element.

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increase in lipogenesis was observed in the *THRSP* knockout mouse, this contradiction could be due to incomplete gene deletion or overcompensation by alternative pathways (Zhu et al., 2001). Homodimers of *THRSP* interact with and activate chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) in promoting transcription of L-type pyruvate kinase (*L-PK*) through its interaction with specificity protein 1 (Sp1) binding site (Compe et al., 2001). Furthermore, the *THRSP* promoter region contains three thyroid response elements (TREs) that work synergistically and interact with far upstream region (FUR) elements to maximize triiodothyronine (T_3) responses in hepatocytes (Liu and Towle, 1994). Apparently, the human *THRSP* promoter responds more robustly to T_3 than glucose, while the rat *THRSP* promoter region is more responsive to glucose than T_3 (Campbell et al., 2003).

Chicken Spot 14 (*THRSP*) was first identified by microarray analysis as a differentially regulated EST (pat. pk0032.c9.f) in livers of chickens divergently selected for fast or slow growth rate (Cogburn et al., 2000, 2003b). A related EST was revealed by differential mRNA display in liver of genetically fat and lean chickens and subsequently mapped to 1q41–44 (Carre et al., 2001). This chromosomal region in chickens also harbors quantitative trait loci (QTL) for skin fatness (Ikeobi et al., 2002) and abdominal fatness (Lagarrigue et al., 2003). Furthermore, expression of *THRSP* mRNA is regulated by thyroid hormone status in broiler chickens (Wang et al., 2002).

In this paper, we describe the identification, characterization and expression of duplicated, polymorphic Spot 14 (*THRSPα* and *THRSPβ*) genes in the chicken. We also report an association of the *THRSPα* locus with abdominal fat traits in a Leghorn × broiler resource population.

2. Materials and methods

2.1. Chicken EST assembly and DNA sequence analyses

The in silico cDNA sequence of *THRSP* was assembled from chicken EST sequences generated from two international chicken EST projects (Boardman et al., 2002; Cogburn et al., submitted for publication) and those found in public databases (GenBank). Contigs were assembled using CAP3 (Huang and Madan, 1999) with 40-bp overlap and 90% identity; the CAP3 assemblies and a chicken gene index are available on our website (http://udgenome.ags.udel.edu/~cogburn). Contig and unassembled singlet sequences were used in BlastN and BlastX searches for identification of chicken genes.

The in silico cDNA sequence of chicken *THRSP* was also used in BlastN searches against the GenBank chicken genome trace archive deposited by the Washington University Genome Center (http://www.genome.wustl.edu/projects/chicken/). The sequences of the Blast hits and their mate pairs were retrieved and used by the CAP3 program to build genomic contigs, which were then used in subsequent BlastN searches. This in silico chromosome walking procedure was repeated five times. The final assembled genomic contigs and singlets were used to blast against our CAP3 database to identify genes in the vicinity of *THRSPα* and *THRSPβ*. To avoid misleading assembly, genomic regions containing multiple chicken repeat 1 (CR1) sequences were carefully inspected.

Table 1
Quantitative RT-PCR (TaqMan) and PCR primers

Gene		Primer sequence	Amplicon size (bp)
18S RNA[a]	Forward	GTGCATTTATCAGACCAAAACCAA	76
	Reverse	GCGATCGGCTCGAGGTTA	
Total *THRSP*[a]	32F	TTCTCGGCCACGCAGAAG	71
	93R	AAGACCCCTCGCAGCAGG	
THRSPα[b]	DeletionF	GCCTCCGTCACCGATCAG	136 or
	DeletionR	CGGTCAGAACCTGCTGCAA	127
THRSPβ	ParalogF	GCGTCCTTCACCGAGCG	151 or
	ParalogR	TGGCTGAGGATCTGCTGCAG	145
NDUFC2	465F	CGTGTGGATGGCAAGATGTT	151
	615R	CAACTCCAGGCTTGCTGCAT	
ALG8	1053F	GCCTTGTTGTTTGTGCGTTG	460
	1203R	AAATGCCCTGTGGTTGTCAGA	

[a] These primer sets were used in TaqMan real-time qRT-PCR analysis.
[b] Determines both *THRSPα* and *β*.

2.2. PCR conditions

Generally, the PCR mixture contained 50 ng of template and 0.2 μM each primer (Table 1), 0.2 mM dNTP with 2 U Taq DNA polymerase and 1.5 mM $MgCl_2$ in 20 μl final volume. PCR was performed for 35 cycles of 45 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C after an initial denaturation at 95 °C for 2 min and a final extension for 7 min. Due to the high GC content, ThermalAce PCR kit (Invitrogen; Carlsbad, CA) was used to amplify fragments of *THRSPβ*. Thermal cycles are essentially the same for *THRSPβ* as those described above, except that the denaturation step was at 98 °C.

2.3. Analysis of two chicken BAC clones

Two chicken BAC clones (**65J23** and **94A1**) which were positive for chicken *THRSP* (Carre et al., 2001) were obtained from the Texas A&M University BAC Center and BAC DNA prepared using the Large Construct Kit (Qiagen). The primers for chicken NADH dehydrogenase (*NDUFC2*) and glucosyltransferase (*ALG8*) were designed from in silico cDNA sequences (**UD** CAP3 **Contig_7797.2** and **Contig_3078.1**, respectively) which correspond to these chicken genes (see Table 1).

2.4. RNA isolation and real-time quantitative RT-PCR

Tissues of interest were taken immediately after cervical dislocation, snap frozen in liquid nitrogen and stored at

− 80 °C until extraction of RNA. Total RNA was extracted using an RNeasy midi kit (Qiagen; Valencia, CA) and its concentration determined by reading the optical density at 260 nm. Samples were diluted in RNase-free water to a concentration of 20 ng/μl and stored in a 96-well plate at − 80 °C. Real-time quantitative RT-PCR (qRT-PCR) was performed with a PRISM® 7900HT Sequence Detection System (TaqMan®) (ABI; Fullerton, CA). Primers were designed using Primer Express 2.0 software (ABI). The QuantiTech SYBR green RT-PCR kit (Qiagen) and gene-specific PCR primers (Table 1) were used in a 20-μl reaction following protocols recommended by the manufacturer. The concentration of total RNA in each sample was ensured by analyzing 18S RNA by qRT-PCR, which showed no significant difference among samples. A standard curve and conversion factor between primer sets 32F/93R (detects both *THRSPα* and *β*) and DeletionF/DeletionR (α specific) were generated using a plasmid from a *THRSP* EST clone (**pgf2n.pk005.j11**) as template, which was diluted to the optimal concentration range (4.26×10^4 to 1.75×10^8 copies per μl) in water containing 20 ng/μl yeast RNA. The template was then amplified following a standard TaqMan qRT-PCR protocol (ABI). The expression of *THRSPβ* in chicken tissue was calculated by taking the difference between total *THRSP* (32F/93R primers) and *THRSPα*-specific (DeletionF/DeletionR primers) measurements.

2.5. Genotyping and trait association analysis

The Iowa Growth and Composition Resources Population (IGCRP) was used to study the association of the *THRSPα* polymorphism with abdominal fat traits. This population was established by crossing a broiler sire (from a commercial broiler-breeder male line) with dams from two unrelated highly inbred lines (Leghorn G-B2 and Fayoumi M15.2). These two inbred lines are more than 99% inbred (Zhou and Lamont, 1999). Two F_1 male offspring of the same sire, one from each genetic cross (F_1 Leghorn and F_1 Fayoumi) were randomly selected and each rooster mated with 20 half-sib F_1 females, producing about 720 F_2 offspring in three hatches. Abdominal fat weight (Fat) was measured and also expressed as a percentage of body weight at 8 weeks of age (%Fat). For genotyping of *THRSPα*, genomic DNA samples were amplified by PCR using a fluorescence forward primer (6FAM-DeletionF) and reverse primer DeletionR. The PCR products were analyzed on an automated DNA sequencer with GeneScan program (ABI) used for calling alleles.

The JMP® program (SAS Institute; Cary, NC) (Sall and Lehman, 1996) was used to conduct the general linear model test for association between genotype and fat traits based on model for the whole F_2 population: $Y = \mu + G + \text{Sex} + \text{Dam}_{\text{random}}(\text{Cross}) + \text{Hatch}_{\text{random}} + e$. Where Y is the dependent variable, μ is population mean, G is genotype, and e is the random error. For analysis of each genetic cross, the statistical model was the same except that $\text{Dam}_{\text{random}}$ was substituted for $\text{Dam}_{\text{random}}(\text{Cross})$, because the crosses were analyzed separately.

3. Results

3.1. Identification of THRSPα and THRSPβ paralogs

An in silico cDNA sequence representing chicken *THRSPα* (Fig. 1A) was assembled from a total of 61 ESTs (**UD CAP3 Contig_8452.1**) found in the UD chicken EST (http://www.chickest.udel.edu/), the British Biotechnology and Biological Sciences Research Council (BBSRC) chick EST (http://www.chick.umist.ac.uk/) (Boardman et al., 2002) and GenBank databases. The *THRSPα* contig sequence is 874 bp and it includes two closely located poly(A) signals in the 3′-UTR. No additional sequence was found at the 5′-end of *THRSPα* by 5′-RACE analysis. Northern blot analysis shows that the *THRSPα* transcript is 1.1 kb (data not shown). The predicted size of the *THRSPα* peptide is either 129 or 132 amino acid (aa) (due to the 9-bp VNTR polymorphism in the coding region) with a molecular weight of either 14.5 or 14.2 kDa and a pI of either 4.61 or 4.53. As predicated by the PSORT II program (http://psort.ims.u-tokyo.ac.jp/), this peptide is localized in the nucleus and has a leucine zipper motif in the C terminus. The predicted chicken *THRSPα* peptide (Fig. 1A) has a low similarity (29% identities; 46% positives) to the human *THRSP* aa sequence (Grillasca et al., 1997) and to a gastrulation specific protein, G12 (33% identities; 45% positives) found in zebrafish (Conway, 1995). When a BlastX search of 1630 chicken protein sequences, derived from complete open reading frames in the UD CAP3 chicken EST assemblies, was made against the non-redundant human protein set in GenBank, the similarity of *THRSPα* was among the weakest 2%.

The chicken *THRSPβ* paralog (Fig. 1B) was identified from a tBlastX search of the chicken UD CAP3 contig database (Cogburn et al., submitted for publication) using *THRSPα* as an electronic probe. The in silico cDNA for *THRSPβ* (**UD CAP3 Contig_8452.2**) was assembled from eight ESTs found in the BBSRC collection. It is 670 bp long with a typical poly(A) signal sequence. The *THRSPβ* cDNA is almost identical to *THRSPα* isoform in the first 230 nt at the 5′-end, which encodes a nearly identical N-terminus. The overall similarity between *THRSPα* and *THRSPβ* paralogs is 70% identical and 79% positive (Fig. 1C). The *THRSPβ* cDNA is extremely GC-rich (74.7%), which makes it a difficult target for cloning and PCR amplification. Similar to the THRSPα isoform, the predicted *THRSPβ* protein is acidic (pI 5.1 or 4.96) with a molecular weight of 14.5 or 14.7 kDa and a leucine zipper motif in the C-terminus.

An additional chicken EST found in the BBSRC database (GenBank accession no. BU440998) has an exception-

A. *THRSPα*

```
                                                    32F
gaggagaggcaaggagtggggccATGGAGCAGTACTTCTCGGCCACGCAGAAGATGGAGCAGGAGGTGATGTTCCCCAGC
                          M  E  Q  Y  F  S  A  T  Q  K  M  E  Q  E  V  M  F  P  S
 93R
CTGCTGCGAGGGGTCTTCCCGCAGGACGGGGCCGACCCAGCCACCGGCGGCCCCGCAGACCTCTACGAGCACTACCAGCT
L  L  R  G  V  F  P  Q  D  G  A  D  P  A  T  G  G  P  A  D  L  Y  E  H  Y  Q  L
                                                 DeletionF
CCTCAAGGCCATCAAGCCCGTGGTGGAGCGAGGCCTGGCCTCCGTCACCGATCAGAGCCCCACCAGCAATGCCGACGCCG
 L  K  A  I  K  P  V  V  E  R  G  L  A  S  V  T  D  Q  S  P  T  S  N  A  D  A  D
                                VNTR
ACACGGCCCCATATGATGGCATAGATGGCATAGATGGGAATCTGGAGGAGCGGCTGTCCCACCACATGAATGGCTTGCAG
  T  A  P  Y  D  G  I  D  G  I  D  G  N  L  E  E  R  L  S  H  H  M  N  G  L  Q
DeletionR
CAGGTTCTGACCGACCTCACCAAAAACACCAAAGCTCTCACCCGGAGGTACAGCCAGATCCTGGAGGAGATCAACCTCGG
Q  V  L  T  D  L  T  K  N  T  K  A  L  T  R  R  Y  S  Q  I  L  E  E  I  N  L  G
                                                    ▼
TGAAGGTCAGAGCAGCTCATGAgcctgcacacggagactccaagggtgatctgacgtttgcagcccagcggcagctttat
 E  G  Q  S  S  S  *
tcctgtgccaagtcccaccaaggaatgtcttctgcacagaccagcacagaggcttggctgtaccatccaagctgccacat
ggccaatcctcccggcaaacctcactgctttgtttcacctccattcccggggattgccttgcagtaggcagggcagaaat
gagcgttcgctgttattgcttcctagaaggcatctgtaaccttgaaacaatgcttttgttctgcatgtgcctagaccacc
tccccactagtttctttgataatgtcaccagttcccaaagtcaatgatctgaaataaaatgcaataataaaatgaaaaaa
aaaactcagtgcggaatttgaaa
```

B. *THRSPβ*

```
                                                    32F
gcgaggcgaagcgcggggccATGGAGCGGTACTTCTCGGCCACGCAGAAGATGGAGCAGGAGGTGATGTTCCCCAGCC
                          M  E  R  Y  F  S  A  T  Q  K  M  E  Q  E  V  M  F  P  S  L
 93R
TGCTGCGAGGGGTCTTCCCGCAGGACGGGGCCGACCCAGCCGCCGACGGCCCCGCGGACCTCTACGAGCGCTACCAGC
  L  R  G  V  F  P  Q  D  G  A  D  P  A  A  D  G  P  A  D  L  Y  E  R  Y  Q  L
                                                 ParalogF                    VNTR
TCCTCAAGGCCATCAAGCCCGTGGTGGAGCGAGGCCTGGCGTCCTTCACCGAGCGCAGCTCCGCCGGCCACGCCGACG
  L  K  A  I  K  P  V  V  E  R  G  L  A  S  F  T  E  R  S  S  A  G  H  A  D  A
CCGACGCCGACGCCGAGGACGCGGCGGCCGCAGCCGACGGGGCGGCCGGCAGCCTGGAGCAGCGGCTGTGCCACCACC
  D  A  D  A  E  D  A  A  A  A  A  D  G  A  A  G  S  L  E  Q  R  L  C  H  H  L
                ParalogR
TGGCCGGGCTGCAGCAGATCCTCAGCCACCTGACCAGGGACACCGCCGCCCTGACGCGCCGCTACAGCCAGATCCTGG
  A  G  L  Q  Q  I  L  S  H  L  T  R  D  T  A  A  L  T  R  R  Y  S  Q  I  L  E
AGCGGATCAGCCCCGGCGACGCGCAGCCCAGCTGGTGAccccgcgcggctccgctcagcgccgcgggacggggcggcc
  R  I  S  P  G  D  A  Q  P  S  W  *
tccgagcggcgccggcagagccgcggagccgtctgcggggccgtcccgcggtgccccgcggctccgccgtgcgctccg
tcctcggagagcgccgcgctgccgcgctgggctcggacggagccgtgcggcgccggcgccttcgggctggatcccgga
gccgcgcagcgctgccctctctcgtgttttctaataaaactcgtgtttttccgcaaaaaa
```

C. Comparison of cTHRSPα and cTHRSPβ

```
                    Domain 1                              Domain 2
α 1   MEQYFSATQKMEQEVMFPSLLRGVFPQDGADPATGGPADLYEHYQLLKAIKPVVERGLASVTDQSPTSN 69
        ME+YFSATQKMEQEVMFPSLLRGVFPQDGADPA  GPADLYE YQLLKAIKPVVERGLAS T++S   +
β 1   MERYFSATQKMEQEVMFPSLLRGVFPQDGADPAADGPADLYERYQLLKAIKPVVERGLASFTERSSAGH 69
                          α-VNTR                Domain 3
α 70  ADADTAPYD---GIDGIDGNLEERLSHHMNGLQQVLTDLTKNTKALTRRYSQILEEINLGEGQSSS 132
        ADAD    D     DG  G+LE+RL HH+ GLQQ+L+ LT++T ALTRRYSQILE I+ G+ Q S
β 70  ADADADAEDAAAAADGAAGSLEQRLCHHLAGLQQILSHLTRDTAALTRRYSQILERISPGDAQPSW 135
      β-VNTR                          Leucine zipper motif
```

al high homology (99% nucleotide sequence identity) to bovine THRSP and probably represents a contaminating bovine cDNA sequence.

3.2. Sequence alignment and structural analysis of an extended Spot 14 protein family

A protein database search has revealed that the *THRSP* family has three structurally related members in chickens and zebrafish, whereas mammals (i.e., human, mouse or rat) have only two members. A sequence comparison shows the structural similarity among Spot 14 (THRSP), the zebrafish gastrulation-specific protein (G12), and the hypothetical human protein (STRAIT11499) for chicken, human, mouse, rat and zebrafish (Fig. 2A). The Spot 14 protein family shares three conserved domains: a highly hydrophobic aa sequence (PSLLRDV) near N-terminus (*Domain 1*), a second hydrophobic region in the middle (*Domain 2*) and the distinct leucine zipper motif (*Domain 3*) in the carboxyl terminus (Fig. 1C).

A phylogenetic analysis shows that a common ancestor of birds, fishes and mammals could have expressed two genes that encode structurally related proteins (Fig. 2B). The *THRSP* protein is found in chickens, humans, rodents and zebrafish (zTC192887). A second member of the *THRSP* protein family found among these animals is represented by orthologs of a hypothetical human protein STRAIT11499, which includes two zebrafish orthologs (G12 and zTC194742). The *THRSP* gene is duplicated in chickens, whereas the gastrulation-specific G12 gene is duplicated in zebrafish.

3.3. Genomic organization

Genomic sequence in the vicinity of the *THRSPα* gene was carefully searched using the strategy described in Section 2.1. A single genomic DNA contig (14.4 kb) that includes the *THRSPα* gene and its flanking region was assembled by CAP3 from 185 chicken genomic shotgun sequences from the trace archive in GenBank (http://www.ncbi.nih.gov/Traces/trace.cgi). The alignment of the *THRSPα* cDNA and genomic sequences shows that this gene contains two exons and one intron. Similar to the human *THRSP* gene, the first exon encodes the entire cTHRSP protein, while exon 2 represents the 3′-UTR. Analysis of about 800 bp in the 5′-flanking region of the *THRSPα* gene shows a TATA box that is 39 bp upstream of the transcription start site.

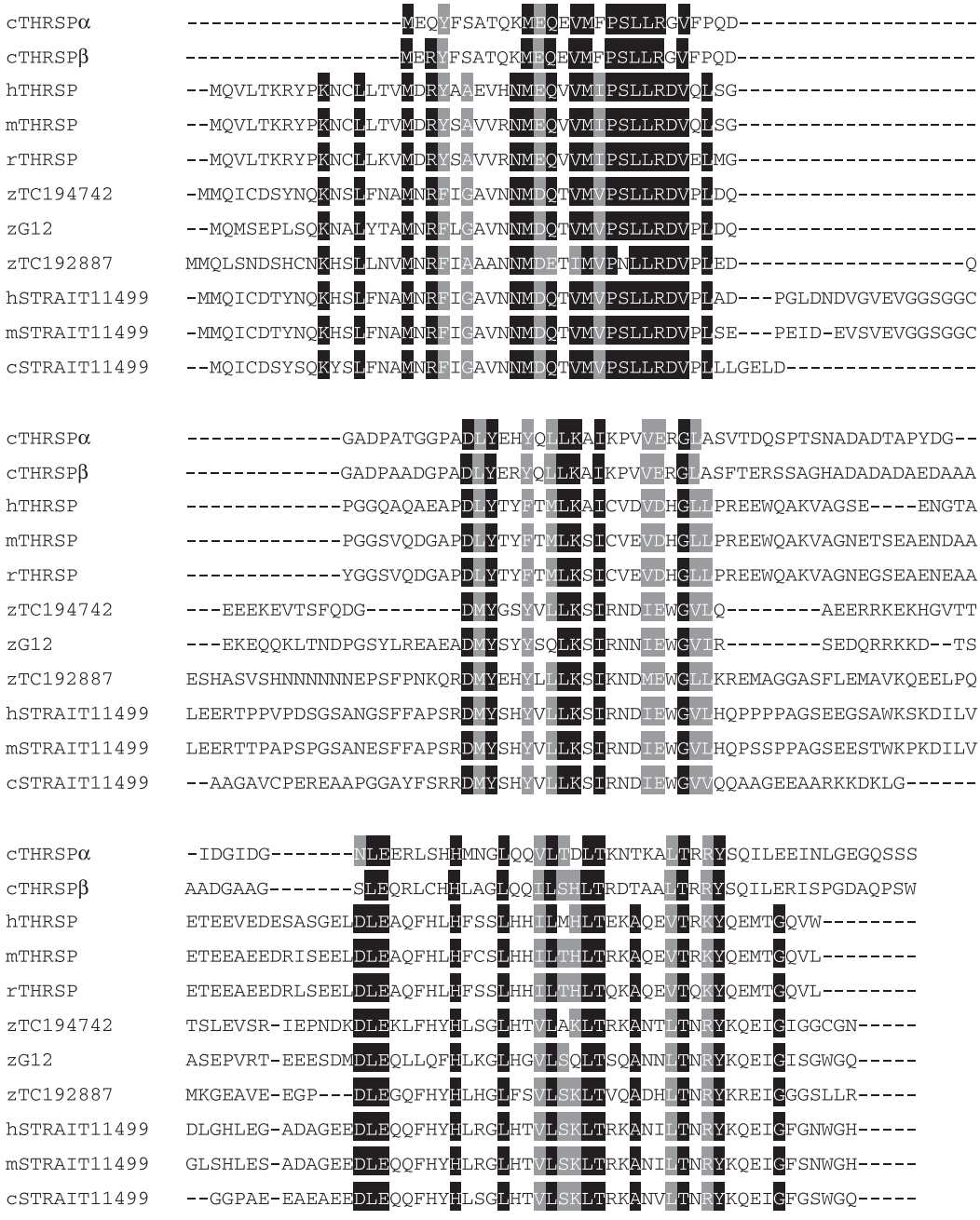
Shotgun sequences from two different clones were found that correspond to the *THRSPβ* gene (GenBank GI no. 253911732 and 298831363), whereas two sequences (GenBank GI no. 253911843 and 298831453), derived from the other end of these two clones, aligned to the *THRSPα* genomic contig. The average size of the shotgun sequencing clones was 4.2 kb; therefore, the chicken *THRSP* paralogs are closely linked. The alignment of these shotgun sequences with *THRSP* paralogs indicates that both genes are transcribed in the same direction. In silico chromosomal walking revealed a synteny group of chicken orthologs of human genes, hypothetical protein MGC2376 (GenBank accession no. XP_133614) and *NDUFC2* (GenBank accession no. AAH07323) in the flanking region of the *THRSP* paralogs. Furthermore, a PCR analysis demonstrates the presence of *THRSPα*, *THRSPβ*, *NDUFC2* and an additional gene glucosyltransferase (*ALG8*; GenBank accession no NP_076984) in two previously identified (Carre et al., 2001) *THRSP*-positive BAC clones (**65J23** and **94A1**) (data not shown). Therefore, this synteny group contains the *THRSPα*, *THRSPβ*, *NDUFC2*, *ALG8* and *MGC2376* genes.

3.4. Expression of THRSP mRNA in chicken tissues

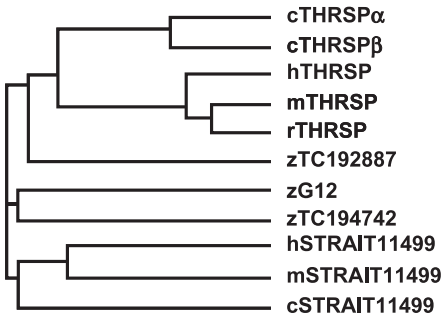
The expression of the chicken *THRSP* genes was examined by qRT-PCR using two primers (32F/93R) (Table 1) that are common to both *THRSPα* and *THRSPβ* (Fig. 1A,B). Among 11 tissues examined, liver had the highest expression level of *THRSP* mRNA, with fat, thymus and ovary expressing lower amounts (Fig. 3A). The chicken *THRSP* genes appear to be predominantly expressed in lipogenic tissue. Direct measurement of *THRSPβ* was not possible by TaqMan analysis (qRT-PCR) because the unique region in *THRSPβ* cDNA is very GC-rich. Therefore, we used an indirect method to examine *THRSPβ* expression in liver and fat tissue (Fig. 3B, C and D). First, we obtained the total *THRSP* mRNA level by using 32F/93R primer pairs; then, the *THRSPα* mRNA was determined using the specific DeletionF/DeletionR primer set. The relative abundance of *THRSPα* was calculated from the difference between total *THRSP* and specific *THRSPα* mRNA levels (Fig. 3B, C and D). The relative abundance of *THRSPα* and *THRSPβ* was examined in liver and abdominal fat of 5-week-old broiler chickens, where the abundance of *THRSPα* was 2- to 3-times greater than that of *THRSPβ*, respectively (Fig. 3B). We have previously found a dramatic increase in chicken total *THRSP* mRNA levels in liver of 1-day-old chicks when compared to late embryos (e16, e18 and e20) (Cogburn et al., 2003a). Therefore, we examined whether the expression of *THRSPα* and *THRSPβ* was differentially regulated during this period. A dramatic increase of 13- to 20-fold was detected in *THRSPα* and *THRSPβ* (Fig. 3C) mRNA levels (respectively) at 1-day post-hatching. Since the expression

Fig. 1. cDNA and predicted protein sequences of chicken *THRSPα* (A) and *THRSPβ* (B). Primer sequences used for PCR are indicated by the bold underlined letters. The predicted leucine zipper motif is shown in bold and the poly(A) signal is underlined. The boxes represent the VNTR region; the shaded portion indicates the missing nt and aa residues in the deletion alleles (α_2 and β_2). The sequence of the 5′-UTR and 3′-UTR (exon 2) of *THRSPα* is shown in lower case letters. The asterisk shows the stop codon. The junction between exons 1 and 2 in *THRSPα* is indicated by the inverted solid triangle. (C) Alignment of chicken *THRSPα* and *THRSPβ* protein sequences using the blastp program. Three functional domains of the putative *THRSPα* and *THRSPβ* proteins are shown in bold letters. The positive aa substitutions are shown by the + sign. The α-VNTR and β-VNTR polymorphisms are shown by the shaded boxes.

A. Conserved domains of THRSP Family



B. Phylogenic Relationships of THRSP (Spot 14) Protein Family



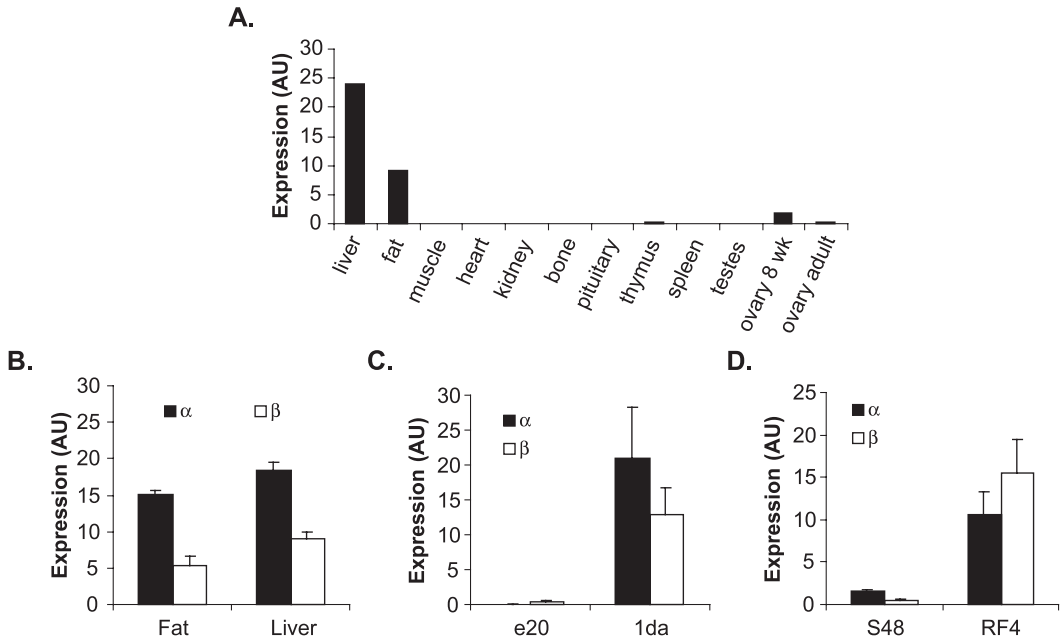


Fig. 3. Expression of *THRSP* transcripts in different chicken tissues. Total RNA (40 ng per reaction) was analyzed by real-time qRT-PCR using TaqMan (ABI) with a universal QuantiTech Sybr Green RT-PCR kit (Qiagen). Primers were designed using Primer Express 2.0 software (ABI). (A) Expression of total *THRSP* in 11 tissues using a common primer pair (32F/93R). Values represent the mean ± S.E.M. of duplicate determinations in arbitrary units (AU). RNA from most tissues was isolated from 5-week-old broiler chickens. RNA from the thymus and epiphyseal growth plate was extracted from 3-week-old broiler chickens. Testes and ovary RNA was isolated from 8-week-old Leghorn chickens; RNA was also collected from the ovary of an adult (1-year-old) Leghorn hen. (B) Expression of *THRSPα* and *THRSPβ* in fat and liver of 5-week-old broiler chickens. (C) Expression of *THRSP* mRNAs in the liver during the peri-hatch period [Day 20 embryos (e20) and 1-day-old (1da) chicks]. Each value represents the mean ± S.E.M. of four embryos and four chicks. (D) The response of hepatic *THRSPα* and *THRSPβ* mRNAs to changes in nutritional state. Liver samples were collected from a fast-growing strain of French (INRA) broiler chickens at 6 weeks of age after a 48-h fast (S48) and at 4-h post re-feeding (RF4) following the 48-h fast (Beccavin et al., 2001). Each value represents the mean ± S.E.M. of four birds.

of *THRSP* responds rapidly to nutritional factors, we also examined whether prolonged fasting or re-feeding (Beccavin et al., 2001) would differentially regulate expression of the hepatic *THRSP* paralogs. *THRSPα* and *THRSPβ* mRNA levels are down-regulated after a 48-h fast and up-regulated at 4 h after re-feeding (Fig. 3D), although the re-feeding response of *THRSPβ* was slightly higher (8-fold increase) than that of *THRSPα* (5-fold increase). Therefore, the transcription of *THRSPα* and *THRSPβ* appears to be controlled by both developmental and nutritional factors.

3.5. THRSP polymorphisms and association of THRSPα alleles with fat traits

The Spot 14 polymorphisms were discovered after detailed examination of EST and shotgun sequence assemblies. Both *THRSP* paralogs are polymorphic which involve VNTRs (ATAGATGGC in *THRSPα* and ACGCCG in *THRSPβ* located near the leucine zipper motif (Fig. 1A,B,C). These polymorphisms result in either the insertion or deletion of the 9-bp (three aa) in the *THRSPα* gene and the insertion or deletion of either 6 or 12 bp (two or four aa) in *THRSPβ*. The 9- and 6-bp polymorphisms have been verified by PCR analysis of broiler, Leghorn and Fayoumi strains (Fig. 4).

The IGCRP (Deeb and Lamont, 2002) was genotyped for *THRSPα* to determine if the polymorphism is associated with deposition of abdominal fat in chickens. In the broiler × Leghorn cross, $THRSP\alpha_1$ homozygotes present a higher fat content than heterozygotes and $THRSP\alpha_2$ homozygotes [Fat ($P<0.048$) and %Fat ($P<0.02$)] (Table 2). Variation of these two alleles accounts for a 3.3-g mean difference in abdominal fat weight between the two homozygous *THRSPα* genotypes. However, there was no associ-

Fig. 2. Protein sequence alignment of the Spot 14 family members (A): *T*hyroid *h*ormone-*r*esponsive *S*pot 14 *p*rotein (THRSP), gastrulation specific protein G12 and the hypothetical protein, STRAIT11499. Protein sequences for chicken [c] *THRSPα* (**UD CAP3 Contig_8452.1**) and *THRSPβ* (**UD CAP3 Contig_8452.2**), human [h] THRSP (**AAH31989**), mouse [m] THRSP (**Q62264**), rat [r] THRSP (**P04143**) and zebrafish [z] (**TC192887**) THRSP were aligned using ClustalW with default parameters and BLOSUM62 scoring matrix. This alignment includes two structurally related proteins: gastrulation-specific protein G12 from zebrafish (**P47805**) and an apparently duplicated G12 protein (**zTC194742**) found in TIGR (http://www.tigr.org/tdb/tgi/zgi/) which show a high degree of structural similarity to the hypothetical human protein hSTRAIT11499 (**AAH19332**), mSTRAIT11499 (**Q9CQ20**) and cSTRAIT11499 (derived from **UD CAP3 Contig_22252.1**). Identical aa residues are shown in black, similar (positive) aa residues are shown in gray and the hyphens denote gaps. A dendrogram showing the phylogenetic relationship among three Spot 14 family members (B): THRSP, the gastrulation-specific protein G12 and hypothetical protein STRAIT11499. The phylogenetic tree was created using the ClustalW program with default settings and the BLOSUM62 scoring matrix.

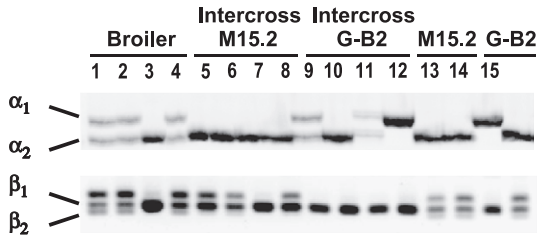


Fig. 4. Evidence of polymorphisms in *THRSPα* and *THRSPβ* genes in Iowa Growth and Composition Resource Population (IGCRP) chickens. Genomic DNA (40 ng) from 15 chickens of mixed sexes, randomly chosen from the IGCRP and contemporary pure founder lines (Broiler; Fayoumi M15.2; Leghorn G-B2), was amplified by PCR with specific primers for either *THRSPα* (DeletionF/DeletionR) or *THRSPβ* ParalogF/ParalogR). The PCR products for *THRSPα* (Allele $\alpha_1 = 136$ bp; Allele $\alpha_2 = 127$ bp) were labeled with ^{32}P-dCTP, separated in native polyacrylamide gel (8%), exposed to a phosphorimager screen overnight and visualized with a PhosphorImager (Storm 840, Molecular Dynamics). The PCR products for *THRSPβ* (Allele $\beta_1 = 151$ bp; Allele $\beta_2 = 145$ bp) were amplified with ThermalAce (Invitrogen) and separated in a 3% agarose gel.

ation between *THRSPα* alleles and abdominal fat traits in the broiler × Fayoumi cross.

The *THRSPβ* locus was not examined on a large scale due to difficulties in PCR amplification of a very GC-rich target sequence.

4. Discussion

In this paper, we have described the discovery, characterization and expression of duplicated, but distinct, *Spot 14* (*THRSPα* and *THRSPβ*) genes and VNTR polymorphisms in *THRSPα* and *THRSPβ*. The *THRSPα* locus is associated with abdominal fat traits in a broiler × Leghorn resource population. The phylogenetic analysis of the *THRSP* protein family among mammals, birds and fishes shows that the chicken *THRSP* paralogs belong to the *THRSP* ortholog group, while zebrafish G12 and TC194742 belong to the *STRAIT11499* ortholog group. This *THRSP* synteny group, containing orthologs of *MGC2376*, NADH dehydrogenase (*NDUFC2*) and glucosyltransferase (*ALG8*), is conserved among chickens, rats, mice and humans, where they are located on GGA1q41–44, RNO1q32–33, MMU7D3-E1 and HSA11q13.5, respectively. Our studies clearly show that a duplication of the *THRSP* gene appeared after the divergence of mammals and birds. Gene duplication is a common process in genome evolution (Tatusov et al., 1997), where each copy of the duplicated genes acquires different mutations that could lead to altered function. One copy of a duplicated gene usually shows a faster rate of evolution (Zhang et al., 2003). *THRSPβ* is more similar in aa sequence to mammalian *THRSP*. However, chicken *THRSPβ* has an unusually high GC content, a feature that is not found in either human or mouse orthologs. It is interesting to note that the flanking *NDUFC2* gene in chicken is also highly polymorphic. Alignment of 22 ESTs and chicken genomic trace sequence reveals two alternatively spliced isoforms and two polymorphic sites in chicken *NDUFC2*. One site is located in the 5′-UTR and involves a GCC repeat, whereas the other polymorphic site is located in the 3′-UTR and involves 4 bp (ATAA).

Although the *THRSP* aa sequence is weakly conserved in vertebrates, an alignment of all members of the *THRSP* family clearly shows three conserved domains (Fig. 2). The leucine zipper motif, which could confer DNA binding ability for *THRSP*, is most evident in chicken paralogs rather than in the mammalian orthologs. This motif and the first hydrophobic domain are required for homodimerization of *THRSP* (Cunningham et al., 1997). The middle hydrophobic domain could mediate the interaction of *THRSP* with other factors.

Expression of the murine *THRSP* has been extensively studied in liver and adipose tissue, where nutritional and hormonal factors intricately regulate its expression (Clarke et al., 1990; Jump et al., 1994; Liu and Towle, 1994). The mammalian *THRSP* promoter contains multiple elements that respond to thyroid hormone (TRE) (Liu and Towle, 1994), carbohydrates (ChoRE) (Koo and Towle, 2000) and sterols (sterol response element, SRE), particularly SREBP-1c (Jump et al., 2001). In chickens, T_3 treatment up-regulates hepatic expression of *THRSP* mRNA (Wang et al., 2002) The expression of *THRSP* paralogs is also dramatically increased in liver of newly hatched chicks and of re-fed chickens following a 48-h fast. Thus, the expression of *THRSPα* and *THRSPβ* appears to be controlled by similar *cis*-elements that respond to developmental, hormonal and nutritional factors, as has been described for mammals.

We have consistently found that *THRSP* has the same expression profile of functionally related genes involved in fat deposition in the chicken (Cogburn et al., 2003a,b). In humans, lipogenic breast cancer is associated with amplification of the *THRSP* gene and enhanced long-chain fatty acid synthesis (Moncur et al., 1998). Furthermore, the only evidence that *THRSP* is directly involved in transcription of metabolic genes is its interaction with COUP-TF1 in the

Table 2
Association of chicken *THRSPα* alleles with fat traits in the Iowa Growth and Composition Resource Population (IGCRP)

		α1/α1	α1/α2	α2/α2	*P* value
Whole population	*N*	180	357	165	
	Fat (g)	52.60	49.33	51.13	0.07
	%Fat	3.36	3.18	3.29	0.08
Broiler × Leghorn	*N*	151	194	44	
	Fat (g)	54.14	50.12	50.81	0.048
	%Fat	3.44	3.17	3.23	0.021
Broiler × Fayoumi	*N*	29	163	121	
	Fat (g)	49.11	49.20	51.16	0.58
	%Fat	3.09	3.23	3.33	0.42

The values represent least square means. The number (*N*) of birds per population (or cross) and genotype is indicated.

control of L-PK (a glycolytic enzyme) (Cunningham et al., 1997, 1998; Compe et al., 2001). Although, the exact mechanism by which *THRSP* exerts its action on transcription of lipogenic enzymes has not yet been established, there is considerable support for a role in regulation of the lipogenic pathway in birds and mammals.

In the present study, we have demonstrated the association of the *THRSPα* locus with abdominal fat traits in a broiler × Leghorn cross, but not in the broiler × Fayoumi cross. This discrepancy could be explained by a large difference in genetic background between the Leghorn and Fayoumi breeds. The polymorphism in *THRSPα* explains about 6% of the variation in abdominal fat, which correlates well with the estimate of about 21 polygenes that control expression of the %Fat trait in this population (Deeb and Lamont, 2002). It is particularly interesting that polymorphisms in *THRSPα* and *THRSPβ* involve aspartic acid near the leucine zipper motif, which is critical for homodimerization and transcriptional regulation of lipogenic enzymes (Cunningham et al., 1997). The duplicated *THRSPα* and *THRSPβ* genes, and the interaction of their haplotypes could add additional complexity to dimerization of this putative transcriptional activator in chickens. It could be much more complicated to determine the effect of each individual allele if the two *THRSP* isoforms act differently in controlling fat deposition. Clearly, the duplicated chicken *THRSP* genes (*THRSPα* and *THRSPβ* paralogs) are worthy of further study as potential candidate genes involved in crucial metabolic processes and for genotyping of these alleles in other resource populations and in elite lines of commercial broiler breeders.

5. Conclusions

We have discovered duplicated paralogs of Spot 14 in the chicken, *THRSPα* and *THRSPβ*. The duplicated *THRSP* genes were identified by sequence analysis of contigs assembled from our chicken EST collection and those in public databases. The transcription of *THRSPα* and *THRSPβ* mRNA in liver is controlled by developmental, hormonal and nutritional factors. A computational analysis of *THRSP* proteins has revealed three highly conserved domains in two structurally related proteins from the *THRSP* family (*THRSP* and STRAIT11499) across a number of vertebrates (chicken, zebrafish, rat, mouse and human). Furthermore, an analysis of the genomic sequence has revealed a synteny group, composed of *THRSP* and its flanking genes [NADH dehydrogenase (*NDUFC2*), glucosyltransferase (*ALG8*) and *MGC2376*], that is conserved among chickens, humans, mice and rats. The chicken *THRSP* paralogs are located on Chr1q41–44 near QTL for fatness. Polymorphic alleles involving VNTR were found in the coding region of the chicken *THRSPα* and *THRSPβ* genes. Our study shows that the *THRSPα* locus is associated with abdominal fat traits in a broiler × Leghorn resource population. These observations support a role of *THRSP* in control of lipogenesis and expression of fat traits in the domestic chicken.

Note added in proof

The sequences reported in this paper have been deposited into Genbank under the following accession numbers: AY568628 (*THRSPα1*), AY568629 (*THRSPα2*), AY568630 (*THRSPβ1*), AY568631 (*THRSPβ2*), AY568632 (*STRAIT11499*) and AY568633 (*NDUFC2*).

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