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Original article

Messenger RNA levels and transcription rates of hepatic lipogenesis genes in genetically lean and fat chickens

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Abstract – Levels of body fat content in commercial meat chickens have prompted research in order to control the development of this trait. Based on experimentally selected divergent lean and fat lines, many studies have shown that liver metabolism has a major role in the fatness variability. In order to identify which genes are involved in this variability, we investigated the expression of several genes implicated in the hepatic lipid metabolism. The studied genes code for enzymes of fatty acid synthesis [ATP citrate-lyase (ACL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), malic enzyme (ME), stearoyl-CoA desaturase (SCD1)], for an apolipoprotein [apolipoprotein A1 (APOA1)], and for the CCAAT/enhancer binding protein α (C/EBPα), which is a transcription factor implied in the regulation of several genes of lipid metabolism. The results show that the fat-line chickens display significantly higher hepatic transcription rates and mRNA levels than the lean-line chickens for the ACL, ME and APOA1 genes. This suggests that these genes could be responsible for the phenotypic fatness variability.

chicken / fatness / mRNA / transcription / lipid metabolism

Résumé – Niveaux d'ARNm et taux de transcription hépatiques de gènes de la lipogénèse chez des poulets génétiquement gras et maigres. L'engraissement excessif du poulet de chair a conduit à développer des recherches afin de maîtriser ce caractère défavorable. De nombreuses études, effectuées sur des lignées expérimentales de poulets gras et maigres obtenues par sélection divergente, ont montré que le métabolisme hépatique joue un rôle majeur dans la variabilité de l'état d'engraissement. Dans le but d'identifier les gènes impliqués dans cette variabilité, le niveau d'expression hépatique de différents gènes impliqués dans le métabolisme des lipides a été analysé. Les gènes étudiés codent pour des enzymes de la synthèse des acides gras [ATP citrate-lyase (ACL), acétyl-CoA carboxylase (ACC), synthase des acides gras (FAS), enzyme malique (ME), stéaroyl-CoA désaturase (SCD1)], pour une apolipoprotéine [apolipoprotéine A1 (APOA1)], et pour C/EBPα (CCAAT/enhancer

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binding protein α), un facteur de transcription régulant plusieurs gènes du métabolisme des lipides Les résultats montrent que les poulets de la lignée grasse présentent des taux de transcription et des niveaux de messagers hépatiques des gènes ACL, EM et APOA1 significativement plus élevés que ceux de la lignée maigre Ce résultat suggère que ces gènes pourraient être responsables de la variabilité d'engraissement observée

poulet / engraissement / ARNm / transcription / métabolisme des lipides

1. INTRODUCTION

Commercial strains of broiler chickens contain between 15 and 18% of their body weight as fat, mainly stored in the abdominal fat tissue. This proportion has been increased over the last few years as a result of intensive selection on growth rate. Excess adipose tissue decreases both feed efficiency during the rearing period and lean meat yield in processing. The resulting economic impairment has prompted many investigations in order to understand the metabolic and genetic mechanisms controlling fat deposition in broilers and to develop practical methods for its control For that purpose, several lean and fat chicken lines have been obtained by divergent selection on various criteria including very low density lipoprotein (VLDL) concentration in plasma [35] or abdominal fat weight [5,22].

The present study is based on a comparison between fat and lean lines selected on abdominal fat weight (FL and LL, respectively) [23]. Metabolic mechanisms leading to fatness have been partly elucidated in these experimental lines. Previous studies have shown that differences in feed consumption and metabolic utilisation of energy between lines cannot account for the difference in adipose tissue weight [23]. Fat chickens exhibit a higher rate of hepatic lipogenesis than lean chickens, but the differences between lines are not always statistically significant due to the large individual variation within lines [27,30, 31]. The sum of hepatic activities of all four NADPH-generating enzymes has been shown to be significantly higher in the FL [1], whereas malic enzyme (ME) alone has a higher but not statistically significant activity in the FL [26] A significantly higher stearoyl-CoA desaturase (SCD1) activity has been observed in the FL [25,26] as well as a higher activity of acetyl-CoA carboxylase (ACC), but these activities were not statistically significant [26]. Plasma lipoprotein concentrations have been found to be higher in the FL compared to the LL [13, 16,25], resulting from a higher rate of triglyceride secretion from the liver [27]. Concerning lipid uptake by peripheral tissues, lipoprotein lipase (LPL) has been reported to play only a minor role in the regulation of fatness [24] since the higher LPL activity in adipose tissue from the fat chickens results mainly from cell hyperplasia rather than from a higher intrinsic activity of individual adipocytes [15]

The purpose of the present study was to determine which genes, among those involved in liver lipid metabolism, display altered hepatic expression as a result of the selection process. A previous study has revealed a few genes with levels of hepatic mRNA that differ between FL and LL chickens [10]. In the present analysis, the hepatic transcription rates of some of these genes, as well

as some others, were quantified in order to lead the investigation to the gene level itself. The mRNA levels were also analysed. The studied genes code for lipogenesis-related enzymes [ATP citrate-lyase (ACL), which produces acetyl-CoA, the substrate of fatty acid synthase; ACC and fatty acid synthase (FAS), which catalyse fatty acid synthesis *per se*; ME producing NADPH, which is a cofactor of FAS], as well as for proteins implied in fatty acid processing and secretion [SCD1, apolipoprotein A1 (APOA1)] The CCAAT/enhancer binding protein α (C/EBPα) was also taken into consideration. This transcription factor of the leucine zipper family has been described as a regulator of liver and adipocyte gene expression [34], functioning notably as a trans-activator of the SCD1 gene promoter [8]. As a result, several genes display higher transcription rates and mRNA amounts in the FL, suggesting they could be responsible for the phenotypic variability.

2. MATERIALS AND METHODS

2.1. Animals

Male chickens (*Gallus domesticus*) from two strains divergently selected for high and low abdominal fat content [fat line (21 animals) and lean line (17 animals), respectively] [23] were provided *ad libitum* access to a standard commercial diet (weight percentage composition: protein 20, lipids 2.5, carbohydrates 57.5, minerals 6 and moisture 14) ("label" diet, Guyomarch'Cie) until they were 56-day-old. All the animals were killed in a fed state. After killing, the individual weights of body, abdominal fat tissue and liver were recorded. A part of the liver was used directly for run-on assays while the rest was cut into small pieces, frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until RNA extraction

2.2. Isolation of RNA and RNA blotting

Total RNA was extracted from the liver samples according to Chomczynski and Sacchi [7]. Northern blot analyses were performed with 10 μg of total liver RNA from each animal as described [10]. For each probe, 2 membranes, each carrying all the RNA samples from 21 fat and 17 lean chicken livers, were treated independently to take into account the experimental variability.

2.3. Probes and hybridization

Five of the probes used were cloned from the chicken. The full-length cDNA clones for ME (1.2 kb, [2]) APOA1 (1 kb, [11]), and C/EBPα (1.3 kb, [6]) were kindly provided by the authors who cloned them. The partial cDNA clones for SCD1 (1.2 kb) (EMBL X60465) and ACL (620 bp) (EMBL AJ245664) were cloned in the Rennes laboratory. A probe composed of part of the mouse 18S ribosomal RNA (515 bp, [28]) was also used. Probe labelling and hybridization were performed as described previously [21].

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) analyses

Relative quantification of ACC and FAS mRNA was performed after RT-PCR as described previously [21]. This was because the weak expression of ACC mRNA and the large size of FAS mRNA gave rise to poor hybridization signals For each gene and all the animals, two independent RT-PCRs and gel electrophoresis were carried out

2.5. Nuclei isolation and run-on transcription analyses

Run-on assays measure the rate of *in vitro* transcription starting again after nuclei isolation. During this process, r[α^{32}P]UTP is incorporated into the newly synthesized RNA which are used as probes to hybridize cDNA sequences, corresponding to the studied genes, dotted on membranes in large molecular excess.

For each animal, a 4-g sample of liver was cut into small pieces and homogenized in 20 mL of lysis buffer (8 mM Hepes pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 2 M sucrose, 10% (v:v) glycerol, 1 mM DTT, 0.75 mM spermidine, 0.15 mM spermine) The nuclei were then purified twice by centrifugation through a 2 M sucrose cushion, each for 1 h at 100 000 $\times$ g and 4 °C. The isolated nuclei were resuspended in a washing buffer (50 mM Tris-HCl pH 8, 140 mM KCl, 10% (v:v) glycerol, 10 mM $MgCl_2$, 0.5 mM EDTA). The nuclei amount was estimated by optical density after lysis of 10 μL of the nuclei solution in 0.5% SDS (1 mg of DNA corresponds to about 100 $\times$ 10^6 nuclei). The rest of the nuclei were centrifuged for 10 min at 650 $\times$ g and 4 °C The pellet was resuspended in a storage buffer (50 mM Tris-HCl pH 8, 140 mM KCl, 40% (v·v) glycerol, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM PMSF, 1 mM DTT) and stored at −80 °C.

A volume corresponding to 50 $\times$ 10^6 nuclei was added with 1 mM rATP, rGTP, rCTP, 10 mM creatine phosphate, 10 $\mu g \cdot mL^{-1}$ creatine phosphokinase, 1 500 $U \cdot mL^{-1}$ RNAsin, 4 mM $MnCl_2$, 100 μCi of r[α^{32}P]UTP (3 000 $Ci\,mmol^{-1}$; Amersham, Les Ulis, France) and 2 μL of heparin, and adjusted to 200 μL with 50 mM Tris-HCl pH 8, 140 mM KCl, 10 mM $MgCl_2$, 0 5 mM EDTA, 0.1 mM PMSF and 1 mM DTT. The reaction was incubated for 30 min at 30 °C, for a further 30 min at 37 °C in the presence of RNase-free DNase (15 UI), and finally for 30 min in the presence of proteinase K (25 μg). Total RNA was then extracted with the RNAxel Kit (Eurobio, Les Ulis, France) and counted.

For each gene studied, a DNA fragment (2 pmol each) was dotted onto filters. These DNA fragments were amplified from the plasmid carrying cDNA (ACL, ME, SCD1, APOA1 and C/EBPα; see 2.3) or by RT-PCR (ACC, FAS; see 2.4). The filter-bound DNA was prehybridized for 5 h at 42 °C in 50 mM Tris-HCl pH 7.5, 0.1% (w:v) NaCl pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 5 mM EDTA, 1 $\times$ SSC, 50% formamide and 150 $\mu g \cdot mL^{-1}$ herring sperm DNA Hybridizations were performed at 42 °C for 72 h using an equal amount of radioactivity from each individual transcription reaction for each

blot. The membranes were washed at 65 °C for 30 min successively in 1 × SSC and 0 1% SDS, and then in 0.5 × SSC and 0 1% SDS. They were treated with RNase A (10 $\mu g \cdot mL^{-1}$) at 37 °C for 30 min, and finally washed in 0.2 × SSC and 0.1% SDS at 65 °C for 30 min. The animals were separated into three groups, and the respective samples were independently submitted to *in vitro* transcription and hybridization assays. The values recorded for each group constituted one repetition.

2.6. Signal treatment and statistical analyses

Hybridizations were assayed and quantified on a Storm instrument (Molecular Dynamics, Bondoufle, France) using a scale of 10^5 grey levels. Each northern blot was first probed with one of the studied probes and then with the 18S probe used as a control of RNA loading; then the mRNA amounts were expressed as arbitrary grey level units relative to 10 μg of total liver RNA, corrected for the possible variation of the quantity loaded onto the membrane by multiplying it by the ratio of the mean 18S signal (calculated for the whole membrane) to the individual 18S signal. For each gene, the relative transcription rate was expressed as the hybridization intensity of the relevant blotted cDNA because the same amount of labelled RNA was used in each hybridization.

The RT-PCR signals (Ethidium Bromide levels) were quantified on digitized images using a CCD camera (Appligene, Illkirch, France) and the Densylab™ 2.6.6 software (Microvision Instruments, Evry, France) Values were expressed as arbitrary units (256 grey levels), corresponding to the specific signal corrected for background staining

For each quantified parameter (mRNA level and transcription rate), the line effect was tested by an analysis of variance using the SAS General Linear Model procedure [32]. The repetition effect was taken into account in the transcription rate analysis. Since no line × repetition interaction was observed, only those results related to the line effect are presented here.

3. RESULTS

3.1. Animal performances

Animal performances for each line, as well as the statistical difference between lines, are reported in Table I. As expected, the difference in abdominal fat content was highly significant. FL chickens had three to four times the amount of adipose tissue compared to the lean animals. In this sample, contrary to previous studies, the body and liver weights of the LL chickens were found to be higher than those of the FL. However, the liver weight to body weight ratio did not differ between lines, which means that the lipogenesis potential of the liver remains similar in both lines.

Table I. Mean and SEM values of animal performances for nine-week-old male chickens from lean (LL) and fat (FL) lines.

	FL (21)	LL (17)	P
BW (g)	2 295 ± 59	2 518 ± 45	0.006
AF (g)	55 2 ± 4 6	13 3 ± 1 9	3×10^{-9}
(AF/BW) × 100	2 3 ± 0.2	0 5 ± 0 1	2×10^{-10}
LW (g)	50 7 ± 1 9	55 6 ± 1.3	0.05
(LW/BW) × 100	2.2 ± 0.1	2.2 ± 0.1	0.93

The number of chickens is given in brackets; BW = Body Weight, AF = Abdominal Fat Tissue Weight; LW = Liver Weight, P = Probability value of between-line F test

3.2. Comparison of liver specific mRNA levels and transcription rates between fat-line and lean-line chickens

The between-line comparisons with respect to transcription rates are reported in Table II.A. Three classes of transcription rate were identified, according to the probability value of the F test related to the line effect. One class included the ACL, ME and APOA1 genes, which display significant differences of transcription rates between lines, the higher values being in the FL. The fat to lean ratios ranged from 1 65 to 2.26. The second class displayed ACC and SCD1 genes with slightly but not significantly higher transcription rates in the FL. The other transcription rates (FAS and C/EBPα) did not differ between lines

The results of the mRNA amounts are reported in Table II.B. The results were comparable to those for transcription rates. The mRNA amounts were significantly higher in the FL for the ACL, ME and APOA1 genes. They were higher in the FL but not significantly so for the ACC and SCD1 genes. No difference was observed for the FAS and C/EBPα genes.

4. DISCUSSION

The aim of this study was to investigate the expression of genes which could be related to the variability of adipose tissue weight. For this purpose, we compared the hepatic transcription and mRNA levels of several genes involved in the lipid metabolism between two lean and fat chicken lines.

Phenotypic traits recorded in the studied sample were somewhat different from previous experiments [10,26,27] in which no between-line differences were recorded for body weight nor for liver weight. These results could be due to a selection relaxation over several generations. However, this new sample remains relevant for the present investigation because of the very large between-line difference in abdominal adipose tissue weight, which could not result from body size difference.

With regards to gene expression, three genes (ACL, ME and APOA1) exhibited significantly higher transcription rates and mRNA levels in the FL

Table II. Mean and SEM values of gene transcription rates and mRNA amounts in the liver of nine-week-old chickens from lean (LL) and fat (FL) lines.

A. Transcription rates				
Gene	FL	LL	FL/LL	*P*
ACL	7 288 ± 1 278	3 701 ± 1 309	1.97	**0.06**
ACC	5 720 ± 1 228	2 904 ± 1 419	1.97	0 15
FAS	8 491 ± 1 742	5 810 ± 2 114	1.46	0.34
ME	26 174 ± 2 502	15 893 ± 2 900	1.65	**0.01**
SCD1	45 827 ± 6 600	30 578 ± 7 490	1.50	0.14
APOA1	24 212 ± 3 489	10 685 ± 3 880	2.26	**0.01**
C/EBPα	33 941 ± 5 201	28 790 ± 5 901	1.18	0.52
B. mRNA amounts				
Gene	FL	LL	FL/LL	*P*
ACL [a]	70 649 ± 12 068	30 235 ± 7 876	2.34	**0.01**
ACC [b]	53.6 ± 1.6	50 5 ± 1 8	1.06	0.10
FAS [b]	2.0 ± 0 2	1.5 ± 0 3	1.33	0.22
ME [a]	78 339 ± 14 936	31 143 ± 5 629	2 52	**0.009**
SCD1 [a]	266 657 ± 68 641	127 459 ± 32 770	2 09	0 08
APOA1 [a]	8 551 804 ± 1 008 566	5 405 989 ± 650 511	1 58	**0.01**
C/EBPα [a]	47 297 ± 6 012	46 401 ± 7 857	1 02	0 93

A. The transcription rates were estimated by run-on experiments and expressed as the intensity of the specific band (arbitrary units).
B. The relative mRNA quantifications (arbitrary units) were performed after hybridization experiments [(a)] or by RT-PCR [(b)] Means and SEM of one experiment are presented. The experiments were repeated twice for each gene with similar results.
ACL = ATP Citrate-Lyase; ACC = Acetyl-CoA Carboxylase; FAS = Fatty Acid Synthase; ME = Malic Enzyme; SCD1 = Stearoyl-CoA Desaturase, APOA1 = Apolipoprotein A1, C/EBPα = CCAAT/Enhancer Binding Protein α; FL/LL = ratio of the mean value in the FL to that in the LL; *P* = Probability value of the F test

compared to the LL. These data suggest that the already recorded increase in hepatic lipid synthesis and secretion results from an increased amount of available transcripts and that the genes identified in this study contribute to the fattening variability.

The present result for ACL was in agreement with higher mRNA concentrations or ACL activities in other excessive fatness models, as seen in the chicken [3] or in Wistar obese rats [17]. These changes, resulting from ACL gene expression, could be responsible for an increased rate of substrate flow for lipogenesis.

The FAS enzyme needs NADPH as a cofactor. In birds, most of the NADPH required for this process is provided by ME [12] and the activity of ME in the liver is positively correlated with the rate of *de novo* fatty acid biosynthesis [36]. The present results concerning the ME gene expression are consistent with the slightly although not significantly higher ME activity in 11-week-old FL

compared with LL chickens [26] Similarly increased hepatic ME mRNA levels have been found in ob/ob mice [9], suggesting a relevant role of the ME gene in fatness variability.

APOA1 mRNA levels were found to be significantly higher in the FL chickens, in agreement with previous results [10], as well as the transcription levels. APOAI is the major apolipoprotein of avian High Density Lipoproteins (HDL) [18], whose plasma concentration is also higher in the FL compared to the LL [14]. HDL are mainly known for their involvement in cholesterol reverse transport, but they are also considered to have an indirect but important role in the catabolism of triglyceride-rich lipoproteins (VLDL), which is increased in the fat chickens along with the VLDL plasma concentration [15]. Besides, the chicken VLDL are primarily dedicated to triglyceride transport from the liver to other tissues; they also contain APOA1, unlike the VLDL of mammals [13,20]. In mammals, apolipoprotein E (APOE) plays a major role in the lipoprotein metabolism, being recognized by a specific receptor (APOB/E receptor) on hepatocyte membranes. Evidence for such an APOE has not yet been found in birds, but it has been suggested that APOA1, whose sequence is partly similar to that of the human APOE, may fulfill the APOE role in chicken lipoprotein catabolism [4], and thus also in fat deposition

The other studied genes displayed little or no differences between the fat and lean chickens As for the fatty acid synthesis itself, no significant between-line differences in gene expression were found for ACC and FAS, even if the ACC values were higher in the fat line. Higher ACC activity, but beneath the significance level, has also been described in the FL [26]. In this previous study, it is likely that a large individual variation may have obscured possible between-line differences for this enzyme and its gene expression. With respect to the fatty acid desaturation, a higher SCD1 activity has been observed in FL chickens [25,26] as well as higher liver mRNA amounts (Daval, personal communication). In the present study, the FL chickens exhibited higher SCD1 mRNA and transcription levels than LL chickens, although the differences were not significant This result does not allow us to draw any conclusion on the role of the SCD1 gene in fatness variability.

Genes involved in lipid metabolism are known to be regulated in a coordinated manner by various nutrient and hormonal effectors [19,33], thus suggesting some common regulation factors. In the same way, the differences in gene expression observed between lean and fat chickens could result from a regulator common to the studied genes. Among such potential regulators, the gene expression of C/EBPα was investigated here. It did not differ between lean and fat chickens, as observed in the Zucker rat liver when compared to lean animals, although its expression has been seen to increase in the adipose tissue of obese animals [29]. Possible differences in C/EBPα protein content cannot be inferred with certainty from the present study. However, since transcription has been reported as the main determinant of C/EBPα gene expression [37], C/EBPα therefore seems unlikely to play a primary role in the difference of lipogenic gene expression in the liver of FL and LL chickens.

Finally, three out of the seven studied genes showed much higher hepatic transcription levels in FL chickens compared to the LL. This difference continued at the mRNA level These data suggest that the corresponding genes

are actually implied in the fatness variability. However, it remains to be elucidated whether these genes themselves are responsible for some of the fatness variability or whether they merely contribute to differences in metabolic flows that lead to differences in animal fattening.

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