

Growth hormone gene polymorphism in chicken divergently selected for axial skeleton malformation

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ABSTRACT

Chicken growth hormone (cGH), a polypeptide hormone synthesized in and secreted by pituitary gland, is involved in a variety of physiological functions such as growth, body composition, egg production, aging and reproduction. Two breeds of chickens selected for high and low incidence of scoliosis, were analysed for polymorphisms in the growth hormone gene (GH). The polymorphism of GH gene (PCR-RFLP) was analysed in 30 chickens each breed, Rhode Island Red and Sussex. It was observed five RFLPs in GH gene, three at *MspI* sites in the intron 1 of cGH gene and two *MspI* sites in the intron 4 of cGH gene. The selection for axial skeleton malformation appears to have affected the frequency of GH alleles. It was found differences in allelic distribution between the affected (H) and unaffected (L) lines. Whereas none of the alleles was observed as specific to H or L lines for both breeds. The experimental material examined constitutes a valuable source for identification of real genes causing skeletal defects.

KEY WORDS: divergent selection, chicken, axial skeleton defects, GH polymorphisms

INTRODUCTION

Skeleton defects are an important problem in poultry flocks, particularly in meat type birds. Reasons of the disorders are multiple and the etiology as well as the pathogenesis is till now poor defined, but the pathological changes of skeleton are hereditary (Mercer and Hill, 1984).

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In the United States the estimated annual losses caused by skeletal problems amount to 80-120 million dollars in broiler chickens and 40 million dollars in turkeys (Morris, 1993). Poultry losses caused by skeletal problems are the result of an increased mortality and number of birds culled (caused also by septicaemia-toxaemia) and by the downgrading of breasts and legs (Sullivan, 1994). Skeletal problems are recognised as one of the four major factors limiting the performance of meat-type birds, because fast-growing broilers, reaching market weights at earlier ages, are considered to have more leg problems and suffer from greater losses than slower-growing birds (Day, 1990; Julian, 1998).

The most important inherited skeletal defects of the axial skeleton are mutations affecting the beak, head, neck and body: cerebral hernia, hereditary exencephaly, crossed beak, short mandible, short upper beak, supernumerary ribs, kyphoscoliosis, spondylolisthesis. The genetic basis of most of these skeletal disorders is still not fully understood (Somes, 1990).

The genetic of an inherited form of scoliosis in chicken was studied by Riggins et al. (1977) and McCarrey et al. (1981). Variation of expression scoliosis is attributed to incomplete penetrance of the major genes, additive effects of minor modifying genes, and primarily to environmental effects and seems to be correlated with the onset of sexual maturity (McCarrey et al., 1981). It was shown that the plasma-free hydroxyproline concentration in scoliotic birds is about twice that of normal chickens, which may reflect an abnormality in collagen metabolism (Lin et al., 1980).

Growth hormone (GH), a polypeptide hormone synthesized in and secreted by the pituitary gland, affects a wide variety of physiological parameters such as growth, egg production, body composition, appetite control, aging and reproduction (Vasilatos-Younken et al., 1999; Putnova et al., 2001). Growth hormone gene polymorphisms have also been observed in other animals. For example, a polymorphism observed in intron 3 of bovine growth hormone (bGH) gene was found to be linked to milk protein content (Lagziel et al., 1999). In fact, regulatory elements have been identified in the intron region of the GH gene in various animals. A glucocorticoid regulatory element (GRE), which may be responsible for the transcriptional control of the human GH (hGH) gene, has been located in the first intron of the hGH gene (Moore et al., 1985; Slater et al., 1985). A pituitary-specific transcription factor, GHF1, which was suggested to be involved in the tissue-specific expression of the GH gene has also been identified in intron 3 of the rainbow trout GH gene (Bernardini et al., 1999). These investigations suggest that introns in the GH gene might play a crucial role in the regulation of GH gene expression.

Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) studies on artificially inseminated (AI) bulls have also revealed an association between GH polymorphism and AI bull reproductive performance (Lechniak et al., 1999).

The chicken GH (cGH) gene is similar to mammalian GH genes, containing five exons and four introns. However, the introns of cGH were significantly larger. The

research on met type chicken using RFLP has shown that the GH gene is highly polymorphic. According to Fotouhi et al. (1993) the selection for abdominal fat appears to have affected the frequency of alleles of the growth hormone gene.

Kuhnlein et al. (1997) analysed 12 non-inbred strains of White Leghorn chicken by PCR-RFLP at three *MspI* sites (PM1, PM2 and PM3) and one *SacI* site (PS1). These sites were located at intron 1 (PM3), intron 3 (PM2), and intron 4 (PM1, PS1), respectively. The author suggested that these alleles, located within the introns, were selected either for an array of egg production traits, resistance to Marek's disease, or resistance to avian leukosis. The GH gene polymorphisms were determined by PCR-RFLP in 28 populations of native Chinese chickens by Ip et al. (2001). Yellow Wai Chow GH gene was characterized by sequencing and was found to have one silent substitution, 31 insertions, and other substitutions spread among the introns (Ip et al., 2001). Recently a novel *MspI* site in the 4 intron of the cGH gene was reported (Nie et al., 2002)

Our objective is to investigate the GH gene polymorphisms in divergently selected lines of chicken for skeletal defects. The allelic composition and frequencies were estimated for each chicken line. The implication of GH in a variety of physiological functions prompted us to search for DNA polymorphisms in the GH gene and test whether the incidence of such polymorphisms is affected by selection for high and low incidence of axial skeleton defects.

MATERIAL AND METHODS

Experimental material

The experimental material consisted of chickens of two breeds: Rhode Island Red (RIR) and Sussex (SX) divergently selected over six generations for high (H) or low (L) incidence of axial skeleton defects in embryos. A description of the material was previously reported by Jaszczak et al. (1999). The divergent selection was performed over six generations on the basis of the analysis frequency of axial skeleton defects (scoliosis) found in dead embryos on day 17-21 of incubation in full families of commercial RIR and SX lines. The frequency of scoliosis in dead embryos in RIR chickens was 40% in H lines and 4.1% in L lines, while in SX chickens, it was 19.5% in H lines and 3.3% in L lines.

Blood samples were collected into vacuum tubes containing EDTA and stored at -20°C. The DNA was extracted by standard methods. DNA concentration was determined spectrophotometrically and DNA was diluted to a final concentration of 0.1 µg/µl.

The analysis of polymorphism in intron 1 and 4 of the GH was performed for 60 individuals - 30 per breed (15 per line with high or low incidence of axial skeleton defects).

PCR-RFLP of intron 1 of GH gene

Intron 1 was amplified by PCR and two primers were

5'- ATCCCCAGGCAAACATCCTC (PM3 forward) and

5'- CCTCGACATCCAGCTCACAT (PM3 reverse).

The PCR conditions used comprised an initial denaturation step of 95°C for 5 min, 35 cycles of denaturation at 94 for 30 sec, annealing at 60°C for 2 min, and an extension step of 72 for 90°C sec.

PCR-RFLP of intron 4 of GH gene

Intron 4 was amplified by PCR and two primers were

5'- CTAAAGGACCTGGAAGAAGGG (PM1 forward) and

5'- AACTTGTCGTAGGTGGGTCTG (PM1 reverse).

The PCR conditions used comprised an initial denaturation step of 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 2 min, and an extension step of 72 for 90°C sec.

The used primers were described previously by Kuhnlein et al. (1997) and gave a predicted product of 776 bp for PCR-RFLP of intron 1 (Ip et al., 2001) and 1170 bp for PCR-RFLP of intron 4 (Nie et al., 2002).

The PCR was carried out in a volume of 25 µl comprising 100 ng of template DNA, 2.5 pmol of each primer, 100 µM of each dNTP, 0.5 unit of DNA polymerase, 10mM tris- HCl (pH 8.8) 1.5 mM MgCl₂, 50 mM KCl and 0.1% Triton X-100. The PCR was performed using a PTC-200 Programmable Thermal Controller (MJ-Research).

The PCR products of intron 1 and 4 (8-12 µl) were digested overnight at 37°C with *MspI* in a reaction mix that contained 1 µl restriction enzyme (about 5 U), 1.5 µl of reaction buffer and analysed by electrophoresis in a 2% agarose gel containing ethidium bromide.

The frequency of alleles and genotypes in analysed regions of gene cGH for 60 animals were than calculated.

RESULTS

PCR-RFLP of intron 1/MspI of gene cGH

A PCR product of expected size, 776 bp, was observed in intron 1 of cGH gene. Ten genotypes were determined by alleles 1, 2, 3, 4 and 5 were identified in intron 1, and three polymorphic *MspI* sites were identified.

Allele 1 had one *MspI* site *a* only, producing two fragments of about 237 and 539 bp. Allele 2 had one *MspI* site *b* only, producing two fragments of 362 and

414 bp. Allele 3 had two *MspI* sites *a* and *b*, in which fragments of 237, 125 and 414, bp were generated. Allele 4 had two *MspI* sites *b* and *c*, in which fragments of 362, 267 and 147 bp were generated. Allele 5 had three *MspI* sites *a*, *b* and *c*, in which fragments of 237, 125, 267, 147 bp were generated (Figure 1).

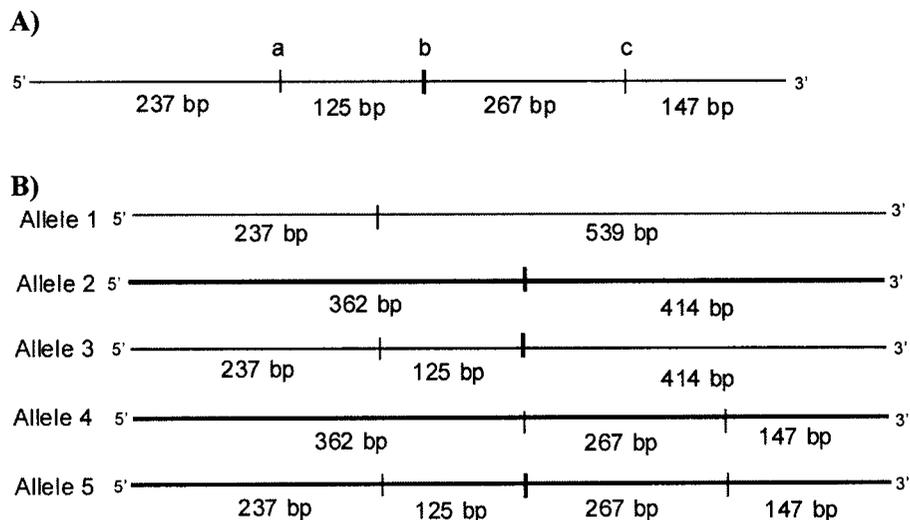


Figure 1. RFLP and mapping of the *MspI* polymorphism in the first intron of the GH gene. A) Restriction map of intron 1. B) Alleles of intron 1 identified in analysed animals

Genotype 1/1 was a homozygote determined by allele 1, Genotype 4/4 was a homozygote determined by allele 4, and Genotype 5/5 was homozygote determined by allele 5. Genotypes 1/2, 1/3, 1/4, 1/5, 2/3, 2/4 and 3/5 were heterozygotes consisting of alleles 1, 2, 3, 4 and 5 (Table 1).

TABLE 1

Genotypes of RFLP polymorphism at the *MspI* site at intron 1 of GH gene.

| Genotypes | Restriction fragments bp |
|-----------|--------------------------|
| 1/1 | 237, 539 |
| 2/1 | 237, 362, 414, 539 |
| 3/1 | 237, 125, 414, 539 |
| 4/1 | 147, 237, 267, 362, 539 |
| 5/1 | 125, 147, 237, 267, 539 |
| 4/2 | 147, 267, 362, 414 |
| 3/2 | 125, 237, 362, 414 |
| 3/5 | 125, 147, 237, 267, 414 |
| 4/4 | 147, 267, 362 |
| 5/5 | 125, 147, 237, 267 |

PCR-RFLP of intron 4/MspI of gene cGH

Two polymorphic *MspI* sites in PCR product of expected size, 1166 bp, were identified. The six genotypes were determined by alleles A, B, and C were identified in intron 4. Allele A had no *MspI* site. Allele B had one *MspI* site *c* only, producing two fragments of 588 bp and 578, which appeared as one band. Allele C had one *MspI* site *d* only, in which fragments of about 416 and 750 bp were generated.

Genotype A/A was a homozygote determined by allele A, genotype B/B was a homozygote determined by allele B, and genotype C/C was homozygote determined by allele C. Genotypes A/B, A/C, B/C were heterozygotes consisting of alleles A, B and C (Figure 2; Table 2).

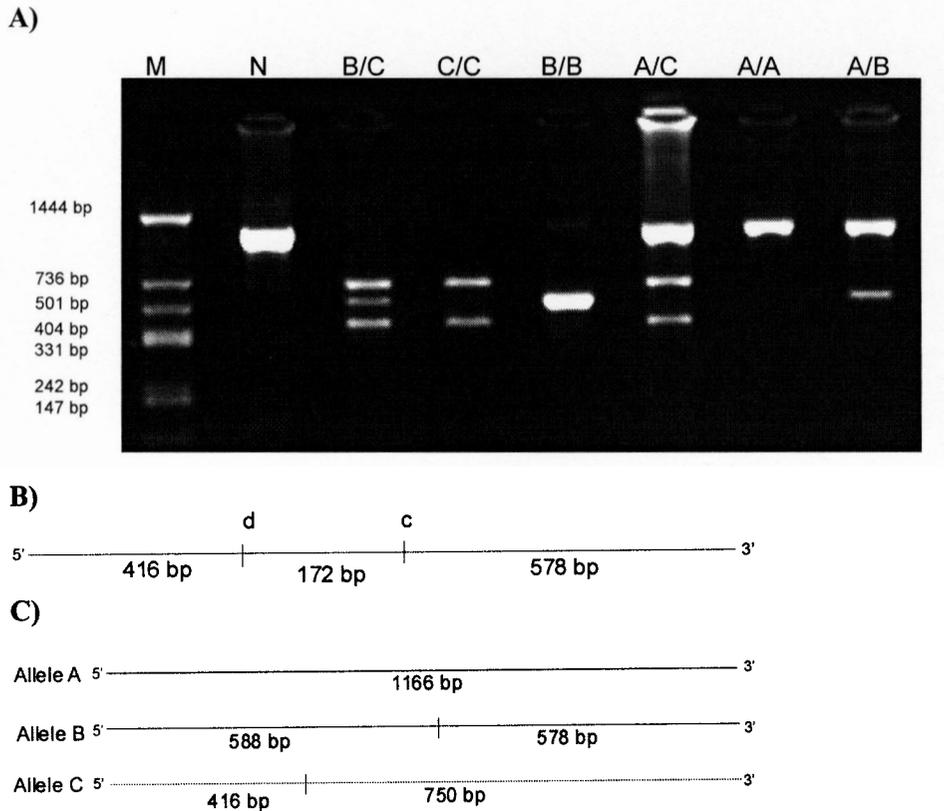


Figure 2. RFLP of the *MspI* polymorphism in intron 4 of the chicken growth hormone gene. A) Profiles of intron 4. M- DNA marker 80-1444 bp (*MspI* digest of pUC19), N-PCR product, genotypes: B/C, C/C, B/B, A/C, A/A, A/B. B) Restriction map of intron 4. C) Alleles of intron 4 identified in analysed animals

TABLE 2

Genotypes of RFLP polymorphism at the *MspI* site at intron 4 of GH gene

| Genotypes | Restriction fragments, bp |
|-----------|---------------------------|
| A/A | 1166 |
| B/B | 578, 588 |
| C/C | 416, 750 |
| A/B | 578, 588, 1166 |
| A/C | 416, 750, 1166 |
| B/C | 416, 750, 578, 588 |

TABLE 3

Frequency of alleles (%) of intron 4 and 1 of GH gene for RIR and SX chicken lines with high (H) or low (L) incidence of axial skeleton defects

| Alleles of intron 1 | Frequency of alleles % | | | | all lines |
|---------------------|------------------------|------|---------|------|-----------|
| | H lines | | L lines | | |
| | SX | RIR | SX | RIR | |
| 1 | 0.0 | 66.6 | 33.3 | 73.3 | 44.0 |
| 2 | 35.7 | 0.0 | 20.0 | 3.3 | 14.4 |
| 3 | 10.7 | 0.0 | 6.6 | 6.6 | 5.9 |
| 4 | 53.5 | 6.6 | 26.6 | 3.3 | 22.0 |
| 5 | 0.0 | 26.6 | 13.3 | 13.3 | 13.5 |

| Alleles of intron 4 | Frequency of alleles % | | | | all lines |
|---------------------|------------------------|------|---------|------|-----------|
| | H lines | | L lines | | |
| | SX | RIR | SX | RIR | |
| A | 57.1 | 13.3 | 73.3 | 6.6 | 37.2 |
| B | 42.8 | 0.0 | 26.6 | 23.3 | 22.8 |
| C | 0.0 | 86.6 | 0.0 | 70.0 | 39.8 |

Allele frequencies of H and L lines of RIR and SX chickens in intron 1 and 4 are shown in Table 3. It was found differences in allelic distribution between the affected (H) and unaffected (L) lines. None of the alleles was observed as specific to H or L lines in both breeds simultaneously.

For the SX breed specific alleles for L lines were identified in intron 1/*MspI* (allele 1, allele 5). For the RIR breed specific alleles for L lines were identified in intron 1/*MspI* (allele 2, allele 3), PCR - RFLP of intron 4/*MspI* (allele B).

Genotype frequencies of H and L lines of RIR and SX chickens in intron 1 and 4 are shown in Table 4. Specific genotypes for H lines were identified only in intron 1/*MspI*: 1/5 - for the RIR breed, 2/3 - for the SX breed. Specific genotypes for L lines were identified in intron 1/*MspI*: 1/2, 3/5- for the SX breed 1/3 - for the RIR breed, and PCR-RFLP of intron 4/*MspI*: B/C - for the RIR breed.

DISCUSSION

Similar to GH genes identified in other mammals, the cGH gene consists of five exons and four introns. It has been reported that the size of the cGH gene is about 3.5 kb (Tanaka et al., 1992; Mou et al., 1995; Ip et al., 2001). RFLPs have been characterized in the introns of cGH gene of White Leghorn and it has been suggested that the alleles identified were linked to egg production traits, resistance to Marek's disease, and avian leukosis (ALV) (Kuhnlein et al., 1997). PCR-RFLPs of the cGH gene were also studied in various populations of Chinese native chickens and it was suggested that an allele present in intron 1 might be linked to laying performance (Ip et al., 2001).

We assumed that our experimental material became fixed as regards scoliosis alleles at all major loci because of inbreeding and selection. It was created from closely related lines (after 5 generations the coefficient of inbreeding amounted to 20.9 and 14.2% in RIR chickens and 14.4 and 11.0% in SX chickens for lines H and L, respectively).

In lines selected for high axial skeletal deformations, a higher mortality of embryos was observed - total losses during the hatching period in H and L lines were 42.5 and 33.0% in the RIR breed and 30.6 and 24.4% in the SX breed, respectively (Jaszczak et al., 1999). The frequency of chromosome abnormalities occurring at the early embryo stages, was significantly lower in the unaffected lines (2.4%) than in lines with developmental spine disturbances (5.7%) (Pryszcz et al., 1999).

The material described was screened for genomic variation on the basis of DNA fingerprinting. An analysis of the representative DNA fingerprinting band patterns led to the identification of specific bands for chicken families with a high or low incidence of axial skeleton defects. The *Hinf*I enzyme/33.6 probe combination produced 30 bands with different frequencies in each family. The average band sharing between lines with a high or low incidence of axial skeleton defects was 0.79, while between families within lines selected for high or low incidence of axial skeleton defects, it was 0.97 and 0.98, respectively (Jaszczak et al., 2001).

As a result of the analysis polymorphism of 15 microsatellite loci in Rhode Island Red (RIR) and Sussex (SX) chickens, divergently selected over six generations for high (H) or low (L) incidence of axial skeleton defects in embryos, 8 specific alleles were identified for H lines and 6 specific alleles for L lines. None of the alleles observed was specific to H or L lines in both breeds simultaneously (Wardęcka et al., 2004).

The results of our study confirm that analysed region of cGH are highly polymorphic. The selection for a high or low level of axial skeleton defects

in embryos, respectively, had different effects on the frequencies of alleles of the analysed loci. A further analysis is needed to check new polymorphisms in chicken growth hormone gene and associations with axial skeleton defects.

Because of similarities in the expression of this disease in chickens and humans, an investigation of the disease loci in chickens may provide useful insights into locating genes affecting adolescent idiopathic scoliosis in humans (McCarrey et al., 1981). Chicken lines selected for high or low skeletal defects constitute a valuable source enabling the identification of real genes causing the effect of interest.

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STRESZCZENIE

Polimorfizm genu hormonu wzrostu u kur selekcyjowanych rozbieżnie na wady embrjonalne szkieletu osiowego

Hormon wzrostu kury (cGH) wpływa na wiele cech ekonomicznie ważnych u tych ptaków, tj. wzrost, masę ciała, reprodukcję i nieśność. Dwie rasy Rhode Island Red i Sussex selekcyjowano rozbieżnie przez sześć pokoleń na wady embrjonalne szkieletu osiowego. Przeanalizowano polimorfizm genu hormonu wzrostu u 30 ptaków każdej rasy, stosując metodę RFLP-PCR. Zidentyfikowano 3 mutacje w intronie 1/*Msp1* i 2 mutacje w intronie 4/*Msp1* genu hormonu wzrostu. Selekcja na embrjonalne wady szkieletu osiowego wpłynęła na różnice w rozkładzie i frekwencji alleli pomiędzy liniami L i H. Wynik ten może być spowodowany bezpośrednim wpływem allelu GH na cechę, bądź odzwierciedla sprzężenie allelu GH z genem determinującym skoliozę. Nie znaleziono alleli specyficznych dla lini H lub L dla obydwóch ras. Materiał doświadczalny jest wartościowym źródłem dla poszukiwania genów warunkujących wady szkieletu osiowego u kur.