Sequence variation in the turkey prolactin promoter and association with incubation behaviour in female turkeys

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ABSTRACT

High concentration of plasma prolactin (PRL) or hyperprolactinemia has long been associated with the expression of incubation behaviour in galliforme species. Incubation behaviour is selected against in commercial turkey production because this maternal behaviour reduces egg production in breeder hens. The identification of sequence variation in the turkey PRL (tPRL) gene and its regulatory region associated with hyperprolactinemia may provide a valid DNA-based marker to further select against the expression of incubation behaviour in commercial flocks. This study was conducted to detect and characterise sequence variation in the turkey PRL gene promoter region. A total of nine PCR-amplified fragments covering about 2.4kb of the tPRL gene promoter region were investigated. The fragments were designated as fragment A_d to H with fragment A_d as the most distal and H proximal from the transcription start site. Single-strand conformation polymorphism (SSCP) analysis showed that five (B, C, E, F and G) out of the nine fragments were polymorphic. Sequence analysis revealed 10 base substitutions, a 12bp deletion, a C insertion and a C deletion at various locations upstream of the tPRL gene. Further investigation has indicated that three out of the 13 polymorphic sites were within potential transcription factor binding motifs. No association was observed between the sequence variation and the overall concentration of plasma PRL. However, further analysis showed that the sequence variation detected within potential transcription factor binding sites was associated with the level of plasma PRL in the incubating hens ($p \leq 0.05$).

Résumé

Les concentrations élevées de prolactine (PRL) plasmatique, ou l'hyperprolactinemie, ont longtemps été associées à l'expression du comportement d'incubation (de couvaison) chez les espèces galliformes. La production commerciale de la dinde vise à sélectionner contre le comportement d'incubation (de couvaison) parce que ce comportement maternel diminue la production d'oeufs chez les dindes reproductrices. L'identification de variations de séquence dans le gène de la PRL associée à l'hyperprolinactinemie fournira peut-être un marqueur génétique pour sélectionner davantage contre ce comportement au niveau des élevages commerciaux. Cette étude a été effectuée pour détecter et caractériser les variations de séquence au niveau de la région du promoteur du gène de la PRL chez la dinde. Un total de neuf fragments amplifiés par la réaction de polymérisation en chaîne (PCR) couvrant 2.4 kb du promoteur du gène de la PRL ont été étudié. Les fragments ont été nommés de A_d jusqu'à H, avec le fragment A_d le plus près et H le plus éloigné du site de transcription. L'analyse SSCP a démontré que cinq (B, C, E, F, et G) des neuf fragments étaient polymorphismes. L'analyse de la séquence a révélé des substitutions de 10 paires de base (pb), 12 pb supprimées, une insertion d'un C et un C supprimé à divers sites avant le gène de la PRL chez la dinde. D'autres études ont indiqué que trois des 13 sites polymorphismes étaient au niveau des motifs d'attachement des déterminants de transcription potentiels. Aucune association n'a été observée entre la variation de séquence et la concentration totale de PRL plasmatique. Cependant, des analyses subséquentes ont démontré que la variation de séquence détectée au niveau des sites d'attachements des déterminants de transcription potentiels était associée au niveau de PRL plasmatique chez les dindes couveuses.

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CHAPTER I. INTRODUCTION

The negative influence of body weight on egg production (Nestor 1985; Nestor 1984; Bacon *et al* 1983; Nestor *et al* 1980) poses a problem of economic importance to poultry producers. This problem is evident in the low rate of egg production particularly in meat-type chicken and turkey strains. The total number of egg produced per hen during the entire galliforme reproductive cycle is dependent on the interaction of several genetic and environmental factors. To increase egg production efficiency, the industry employs intensive selective breeding and management programs to enhance the positive and diminish the negative effects of these factors.

One of the most prominent factors affecting egg production is the expression of incubation behaviour (Lea *et al* 1981; El Halawani 1984b; Sharp 1989a). Incubation behaviour or broodiness is a maternal behaviour expressed by galliforme birds characterised by the desire to incubate the eggs (El Halawani *et al* 1984b) and care for the young. This physiological state is usually accompanied by gonadal regression, which then results in the termination of egg production (Sharp 1989a). Accordingly, the number of eggs produced per hen is determined by the duration of the egg-laying period before the onset of broodiness (Sharp 1980).

Research on the broodiness in domestic galliformes revealed that this behaviour is associated with the anterior pituitary hormone prolactin (Riddle *et al* 1935). Prolactin (PRL) is a multifaceted peptide hormone responsible for the more than 300 distinct actions in vertebrate including osmoregulation in fish, lactation in mammals and the expression of maternal behaviour in birds (reviewed in Boyle-

Feysot *et al* 1998). Early studies on avian physiology have demonstrated the role of PRL in the formation of the brood patch, the development of the crop sac and the secretion of "crop milk" in pigeons (Riddle *et al* 1933). Several behavioural and physical characteristics have been associated with high concentration of plasma PRL in domestic galliformes such as vocal and assertive nest protection (Noll 1989), refusal to leave the nest to feed (Zadworny *et al* 1985a,b), the regression of the ovary and oviduct (Porter *et al* 1991; Sharp 1989a), and other nesting behaviour (Karatzas *et al* 1997; El Halawani *et al* 1984b). Taken together, the expression of incubation behaviour associated with PRL is necessary for the survival of wild galliforme species therefore it persists despite domestication.

The link between PRL and the expression of broodiness is fairly established but the mechanism(s) involved in the process has not been fully understood. Molecular genetics provides novel tools to investigate the role of PRL in avian reproduction on a different perspective. Studies on mammalian PRL gene and its receptor have elucidated some of the mechanisms involved in transcription regulation (Iverson *et al* 1990; Day and Maurer 1989) and signal transduction (Clevenger 1998; Hennighausen *et al* 1997). Research on avian PRL gene has yielded valuable information on the nucleotide sequence of PRL gene in chicken (Hanks *et al* 1989; Watahiki *et al* 1989) and turkey (Karatzas *et al* 1990; Wong *et al* 1991; Kurima *et al* 1995). A recent study on chicken PRL (cPRL) gene revealed the role of its 5'flanking region or promoter in transcription regulation (Ohkubo *et al*, personal communication).

The PRL gene promoter regulates transcription via short DNA motifs that constitute transcription factor binding sites. Transcription factors are small proteins that bind to gene promoters creating a single- or multiple-protein-DNA complexes that aids in the initiation of transcription or modulate the synthesis of RNA in response to the different developmental or environmental signals (Nikolov and Burley 1997). Studies have shown that binding of transcription factors to DNA motifs in the promoter could either enhance or inhibit PRL gene expression in mammals (Day and Maurer 1989; Iverson *et al* 1990), and in domestic fowl (Ohkubo *et al* personal communication). Mutation in one or more nucleotide bases that constitute the consensus sequence of the transcription factor binding site(s) alters its binding affinity and function (Iverson *et al* 1990).

This study was conducted to detect sequence variations in the –2.324kb turkey PRL (tPRL) gene promoter using single-strand conformation polymorphism (SSCP) analyses and restriction fragment length polymorphism (RFLP) analyses. The polymorphisms detected were characterised and 66 birds were subsequently genotyped. Furthermore, the association between the genotypes with their plasma PRL level was also evaluated.

CHAPTER II. REVIEW OF LITERATURE

2.1 Broodiness and the turkey industry

Broodiness, a parental behaviour commonly expressed by galliformes, is characterised by the incubation of eggs and the rearing of the young (El Halawani *et al* 1984). This behaviour generally follows the egg-laying phase of the galliforme breeding cycle. In some wild species both parents are involved in the incubation of eggs as well as protection of their young. For domesticated species however, broodiness is almost entirely expressed by the mother.

Significant behavioural and physical changes can be perceived at the onset of broodiness. Behavioural changes include increased nesting frequency (Zadworny *et al* 1988; El Halawani *et al* 1984), vocal and assertive nest protection (Noll 1989), and the refusal to leave the nest to feed (Zadworny *et al* 1985a,b). Physical changes also observed include ovarian and oviductal regression (Sharp 1989a; Porter *et al* 1991), and the development of brood patches (Riddle *et al* 1933). These physical and behavioural changes are accompanied by neuroendocrinological changes typified by a significant increase in plasma PRL level (Shimada *et al* 1991; Etches and Cheng 1982) and a decrease in plasma LH level (Kuwayama *et al* 1992; Zadworny *et al* 1989). These changes taken together result in the termination of lay and mark the onset of incubation behaviour. Broodiness is critical to the survival of galliformes in the wild thus its expression persists in some species despite domestication (Sharp 1989a).

2.1.1 Brief history of the poultry industry

Man has domesticated animals from the wild and tailored their characteristics to fit his needs. The intention to domesticate differs between mammals and birds. Historical evidence reveal that mammals were generally kept for food and as work animals, while birds were originally domesticated for cultural and religious purposes (Crawford 1995). The earliest record of chicken skeletal remains associated with domestication dating from 5900 to 5400 B.C were found in archaeological sites in China (West and Zhou 1989) while turkey bones dating back from 200 BC to 700 AD were found near Mexico City (Schorger 1966).

The domestic fowl closely resemble four Asiatic junglefowl species but the red junglefowl (*Gallus gallus*) appeared to be the main ancestor (Crawford 1995). Modern chicken breeds and varieties have evolved by natural selection through mutation, genetic drift and migration, as well as artificial selection the propagation of favoured genotypes (*i.e.* aesthetic and food value). The early avian breeding strategies involved the creation of distinct breeds for cultural and religious purposes. The establishment of the poultry industry for the production and marketing of poultry and poultry products for food shifted the breeding strategies from the production of distinct breeds to uniform breeding for commercialisation (Crawford 1990). Two main attributes, growth rate and egg production, were established as commercially viable traits and the subsequent selective breeding for these traits gave rise to the current meat type and layer strains.

The turkey (subspecies *Melleagris gallapavo gallapavo*) which was a native of Mexico and part of South America was introduced to Europe primarily as an exotic food by Spanish explorers (Gascoyne 1989). The pilgrims later reintroduced the species to other regions of North America, which then bred freely with wild turkey species (subspecies *Melleagris gallapavo silvestris*) native to the area (Schorger 1966). The hybrid stock replaced the smaller European-Mexican stock and became known as the American bronze (Crawford 1995).

2.1.2 Breeding structure of commercial birds

As poultry developed into an industry, artificial selection was directed towards economically viable chicken production traits (*i.e.* meat and eggs). Breeding practices shifted from pure breeding to crossbreeding to take advantage of heterosis or hybrid vigour (Crawford 1995). Crossbreeding was used to develop hens capable of producing large numbers of eggs (layers) and meat type chicken strains with accelerated growth rate and marketable carcass quality.

During the development of these breeds, a negative correlation between growth rate and egg production was observed (Chambers 1990). To counter this problem, meat type breeder stocks were selected for specific traits. The male line is selected for growth and carcass quality while the female line is selected for growth and egg production. This combination resulted in the current broiler chicken sold for meat. For layer strains, the increase in the rate of egg production can be attributed to genetics (Gowe and Fairfall 1995) as well as improvements in nutrition and management programs (Crawford 1995). Breeding chicken for egg production almost completely selected against broodiness in breeder stocks. Current layer strains do not

express this behaviour to any great extent (≤6%) and there are no apparent adverse effects on other production traits (Craig and Swanson 1994).

The last 50 years have seen the evolution of turkey production from small-scale backyard farming to a multinational industry (Gascoyne 1989). The current breeding and production structure is similar to that of the breeder hen for broiler production (Melnychuk *et al* 1997). Broodiness however, is more prevalent in turkey compared to chicken and, when left untreated this maternal behaviour is expressed by up to 70% of the birds in most commercial populations. In addition, single trait selection for body weight was demonstrated to decrease egg production in larger commercial strains (Nestor 1984). The opposite is observed in smaller turkey strains, which show a positive correlation between body weight and egg production (Robel 1981).

The negative genetic correlation between growth and reproductive traits limits egg production in strains developed for meat production (Nestor 1984; Chambers 1990) by reducing the persistency of lay and increasing the number of days lost to broodiness (Buss 1989). Accordingly, management strategies are being developed to encourage the persistency of lay and prevent the expression of broodiness in meat type breeder hens.

2.1.3 Management of broodiness in the turkey production

Several poultry management programs are directed toward the detection and treatment of broody hens. The traditional methods of detection of broody hens include physical examination of the birds and daily monitoring of their nesting activity (Noll 1989). Broody birds are subsequently treated by altering the visual,

tactile and auditory environment. These treatments can be applied through one or more of the following: nest deprivation (El Halawani 1980a), the transfer of broody hens into wire cages (Bacon and Nestor 1982) or pens with slotted floors, and flock housing rotation (Noll 1989). The treatments were effective to some degree in interrupting broodiness and increase egg production.

In addition to disrupting broodiness, the treatments were also observed to reduce the circulating PRL concentration. Due to the apparent association between broodiness and PRL concentration, pharmacological treatments were also developed to block the former by inhibiting PRL release from the anterior pituitary gland. The earlier drugs developed for the treatment of broodiness are listed and discussed in Sharp (1989a). These chemicals include gonadal steroids (Haller and Cherms 1961); clomiphene citrate, an anti-oestrogen drug (Renner *et al* 1987; Bedrak *et al* 1983); a chicken anti-PRL serum (Lea *et al* 1981); pimozide, a dopamine receptor blocking agent (Millam *et al* 1980); and p-chlorophenylalanine, a serotonin inhibitor (El Halawani *et al* 1980b).

Since broodiness is controlled by the neuroendocrine system, recently developed vaccines against PRL and the hypothalamic factor that controls its release were tested. The anti-tPRL serum raised in rabbit was effective in reducing the expression of broodiness in passively immunised hens (Crisostomo *et al* 1997). The full prevention of this behaviour however, was accomplished by active immunisation with a more potent GST-tPRL fusion protein (Crisostomo *et al* 1998). Likewise, passive and active immunisation against VIP, a PRL releasing factor (PRF) disrupted incubation behaviour by impairing the hypothalamic mechanism responsible for the

PRL release (Sharp *et al* 1989). A subsequent extension of the egg-laying period which contributed to a 10 to 20% increase in egg production has been observed in treated compared to control birds (Caldwell *et al* 1999). In addition, treated birds show relatively stable plasma PRL level during their reproductive cycle (Youngren *et al* 1994; El Halawani *et al* 1995) and the photo-induced plasma PRL surge was prevented by active immunisation against VIP (El Halawani *et al* 1995).

The association between PRL and broodiness in galliformes has long been established (Riddle *et al* 1935; Saeki and Tanabe 1955). The management strategies developed to increase egg production by reducing PRL release strongly support the participation of the hormone in the initiation and maintenance of broodiness in domestic birds.

2.2 Prolactin and its biological actions

Prolactin (PRL) is a peptide hormone belonging to the GH-PRL family. This family of hormones includes growth hormone (GH), PRL, somatolactin and placental lactogen (PL). The molecular mass of these hormones varies between 20 to 26 kDa and each hormone contains about 200 amino acid residues. The extensive similarity between GH and PRL amino acid sequence, gene structure and their over-lapping function imply that they evolved from the duplication of a common ancestral gene (Cooke and Baxter 1982).

More than 300 biological actions observed throughout the vertebrate classes have been attributed to PRL. Boyle-Feysot *et al* (1998) reviewed and classified the known PRL functions in six categories: 1) water and electrolyte balance, 2) growth

and development, 3) endocrinology and metabolism, 4) brain and behaviour, 5) reproduction, and 6) immunoregulation and protection. Prolactin, like any other hormone, brings about these actions by binding to specific membrane receptors (PRLR). The broad endocrine function PRL may be attributed to the wide distribution of PRLR (Zhou *et al* 1996; Hennighausen *et al* 1997) and augmented by molecular variants (Bedecarrats *et al* 1999a,b,c) and extrapituitary expression and the autocrine/paracrine mode of action (Liu *et al* 1997; Ben-Johnathan *et al* 1996; Boyle-Feysot *et al* 1998).

2.2.1 Prolactin receptors

The PRL specific receptors are single-pass transmembrane proteins that belong to the cytokine receptor superfamily (Hennighausen *et al* 1997; Boyle-Feysot *et al* 1998). In addition to GHR and PRLR, this superfamily includes other genetically related interleukin and hematopoeitic receptors (Boyle-Feysot *et al* 1998; Clevenger *et al* 1998). Members of this superfamily are identified by their conserved disulfide loops formed by two cysteine pairs (Cys12-Cys22 and Cys51-Cys52) and the conserved WSXWS consensus pattern (Trp-Ser-amino acid-Trp-Ser) in the C-terminus extremity of its extracellular domain (ECD) (Boyle-Feysot *et al* 1998; Hennighausen *et al* 1997).

In mammals, the gene encoding PRLR has been mapped in human chromosome 5; p13-14, in mouse chromosome 15; 4.6 cM and in pig chromosome 16 (Vincent *et al* 1998). Multiple forms of PRLR identified in mammals were derived from the alternative splicing of primary gene transcript (Hennighausen *et al* 1997). Three rat PRLR isoforms were detected and these differed in the length of their

intracellular domains. Each variant however, has the conserved region (Box1) proximal to the cell membrane (Boyle-Feysot *et al* 1998; Clevenger *et al* 1998: Boutin *et al* 1988).

In birds, PRLR gene was mapped on the Z chromosome of chicken and Japanese quail (Suzuki *et al* 1999a; Miao *et al* 1999). In addition, the cloning and characterisation of the chicken (Tanaka *et al* 1992) and turkey PRLR gene (Zhou *et al* 1996) revealed that this transmembrane protein is similar in size and amino acid composition to the long form mammalian PRLR. Upon further analysis however, the conserved cysteine loops and WSXWS motif that are singularly expressed in mammals were observed in the two tandemly repeated units (Tanaka *et al* 1992; Zhou *et al* 1996).

2.2.2 Prolactin receptor signal transduction pathway

The conserved Box1, containing a hydrophobic proline rich region in the cytoplasmic tail of PRLR, is associated with proteins of the Janus kinase (JAK) family (Clevenger *et al* 1998; Boyle-Feysot *et al* 1998; Hennighausen *et al* 1997). The JAK family has been postulated to work with the cytokine receptors to transmit hormone signals within the cell. In mammals, the PRLR signalling pathway was modelled from the Nb2 T-cell line (Elberg *et al* 1990) and the activation of milk protein genes in the mammary glands (Hennighausen *et al* 1997). The following summary of PRL signal pathway was based on the reviews by Hennighausen *et al* (1997), Clevenger *et al* (1998) and Boyle-Feysot *et al* (1998).

This model, known as the JAK-Stat signal transduction pathway, is initiated by dimerization of PRLR receptors subsequent to ligand binding. Activation of the

receptors requires the binding of a PRL molecule to two PRLRs forming 1ligand: 2receptor complex. After the formation of this ligand:receptors complex, there is cross tyrosine phosphorylation between JAKs associated with the membrane-proximal cytoplasmic tail of the PRLR and PRLR itself. This event is followed by the formation of a receptor-JAK complex. The phosphorylated tyrosine in the JAKs recruits Stat proteins into the receptor-JAK complex by interacting with the Stat protein SH2 domain. Subsequent phosphorylation of the membrane-bound Stat protein is followed by their dissociation from the receptor-JAK complex. The released phosphorylated Stat proteins dimerize, translocate into the nucleus and activate target genes by binding to specific DNA motifs in their promoter.

The activation of Stat proteins reflects the specificity of a cytokine receptor (Hennighausen *et al* 1997; Boyle-Feysot *et al* 1998). In a recent review, Hennighausen *et al* (1997) indicated that PRL activates Stat 1, 3 and Stat 5 (5a and 5b) and suggested that the Stat5a-Stat5b dimers formed after their phosphorylation bind to PRL response element GAS (TTCNNNGAA) in the promoters of several genes involved in milk production. The presence of Box1 in the chicken and turkey PRLR intercellular domain implies that avian PRL signal transduction follows a similar pathway although the target genes for transcriptional activation have not been identified.

2.2.3 Prolactin isoforms

Some PRL actions could be attributed to molecular variants of the PRL detected in mammals and birds. Most isoforms are observed to originate from post-translation modification by the phosphorylation (Aramburo *et al* 1992; Oetting *et al*

1986) and glycosylation of PRL gene products (Sinha 1995; Bedecarrats *et al* 1999a,b,c). The modulation and distribution of PRL variants appear to be associated with the age-dependent reproductive state in mice (Bollengier *et al* 1996,1989). The variation in the isoform concentration during the different stages of mammalian reproductive cycle suggests that different isoforms might be required at various phases of mammalian reproduction.

Several phosphorylated and glycosylated PRL isoforms were detected in the turkey (Corcoran and Proudman 1991; Bedecarrats *et al* 1999a,b,c). Similar to PRL isoform modulation in mice, the differential expression of turkey PRL isoforms appear to be associated with the reproductive state of the hen (Bedecarrats *et al* 1999a,b,c). Bedecarrats *et al* (1999a,b,c) observed that the ratio between glycosylated and non-glycosylated PRL in the turkey pituitary gland varies between immature, laying, incubating and moulting hens.

The most prominent PRL function in higher vertebrates is its role in mammalian and avian reproduction. In mammals, PRL is important for the proliferation of mammary cells during mammary gland development as well as the transcription of the genes involved in milk production (*i.e.*β-lactoglobulin gene) (Hennighausen *et al* 1997). The expression of incubation behaviour is a consequence of PRL action in galliforme reproduction. Numerous studies have attempted to elucidate the role of PRL in the initiation and/or maintenance of this parental behaviour. To date however, the mechanism(s) involved are still not clearly understood.

2.3 Prolactin and the expression of broodiness in domestic galliformes

The role of PRL in avian reproduction was first observed in the early 1930's when PRL was demonstrated to induced pigeon crop sac development and the secretion of crop milk (Riddle *et al* 1933). Subsequently, Riddle *et al* (1935) reported that the introduction of exogenous PRL (ovine PRL) induced broodiness in domestic fowl. To date, numerous studies have invariably supported the association between PRL and broody behaviour in domestic galliformes. Neuroendocrinological and cytological changes leading to hyperprolactinemia were identified to further elucidate the role of PRL in the expression of broodiness in domestic birds.

2.3.1 Neuroendocrinological mechanisms leading to broodiness

The ability of birds to recognise seasonal changes and synchronise their reproductive cycle with the most favourable condition is critical for the survival of both parents and offspring in the wild (Sharp 1980). Artificial light simulation of daylength has been demonstrated to induce gonadal growth in domesticated birds. Thus, the recognition that the avian breeding cycle is dependent on daylengths has led to the current application of photoperiods to induce early sexual maturation and maintain a 6 to 12-month reproductive cycle in domesticated flocks.

Neuroendocrine studies of photostimulated birds detect an increase in hypothalamo-hypophysial-ovarian axis activity. Photostimulation initiates the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, which in turn activates the secretion of gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormones (FSH) from the pituitary gland. The gonadotropins LH and FSH released from the pituitary gland stimulate the development of ovaries and

maturation of the follicle cells, respectively. The mature ovaries in turn release oestrogen (E) and progesterone (P) and thus, increase plasma concentration of these ovarian steroids. The subsequent increase in the circulating P concentration appears to exert a positive feedback effect on LH (Sharp 1980; Etches 1995). The ensuing ovulation is triggered by a surge in plasma LH level that is followed by oviposition.

A concurrent release of vasoactive intestinal peptide (VIP) from the hypothalamus has also been detected after photostimulation. Studies have shown that this hypothalamic factor induces the release of PRL from the cells of the chicken and pigeon anterior pituitary cell (Hall and Chadwick 1983). Accordingly, the steady rise in plasma PRL level perceived after photostimulation reaches a peak at the onset of incubation behaviour.

The high plasma PRL level following the last oviposition appears to inhibit estradiol release from the ovarian cells (Zadworny *et al* 1989) which in turn, results in ovarian regression and the termination of ovulation. Furthermore, the elevated plasma PRL level appears to have a negative effect on the pituitary release of LH by acting at the hypothalamo-hypohysial axis (Zadworny and Etches 1987). Thus, in the transition from egg-laying phase to incubation phase, a significant rise in plasma PRL level and a drop in plasma LH level is generally observed. The low plasma LH concentration associated with hyperprolactinemia is related to the reduction of hypothalamic GnRHI and GnRHII level in chicken (Dunn *et al* 1996; Sharp *et al* 1989a) and in turkey (Rozenboim and El Halawani 1993).

2.3.2 Cytological changes in the anterior pituitary of a hyperprolactinemic hen

The changes in the pituitary cell content during the different stages of the turkey reproductive cycle may also account for the hormonal changes observed during the onset of broodiness in turkey hens. Immunohistochemical studies of the pigeon anterior pituitary gland revealed the presence of two distinct cell types: growth hormone (GH) immunoreactive cells and PRL immunoreactive cells (Hansen and Hansen 1977). Somatotrophs or GH producing cells are primarily located on the caudal lobe while PRL-secreting cells or lactrotrophs are generally observed on the cephalic lobe of the anterior pituitary gland (Kansaku *et al* 1995, 1994; Berghman *et al* 1992; Jozsa *et al* 1979; Hansen and Hansen 1977).

During the incubation phase, a significant increase in the number of visible PRL immunoreactive cells in the anterior pituitary gland of chicken (Lopez *et al* 1996) and turkey (Ramesh *et al* 1996; 1998) can be detected. The increasing number of lactotrophs were no longer confined to the cephalic lobe of the anterior pituitary gland but were observed in and around, the caudal lobe. The detection of lactotrophs in the caudal lobe of broody hens may be attributed the overflow of increasing lactotrophs (Ramesh *et al* 1996) or to the presence undifferentiated pituitary cells (mammosomatotrophs) in the peripheral boundary between the cephalic and caudal pituitary lobes (Ramesh *et al* 1998). The mammosomatotrophs were presented as pituitary cells capable of GH or PRL secretion depending on the reproductive state of the hen, a condition similar to that observed in mammals.

The role of PRL in the expression of broodiness may be sufficiently supported by the events described but the description of the exact mechanism(s) manifesting this

role has so far eluded investigators. The regulation of PRL release from the pituitary gland provides further insights on the participation of PRL in the expression of this maternal behaviour.

2.4 Regulation of PRL release

Various secretagogues from the vertebrate hypothalamus control PRL release from the anterior pituitary gland. Hypothalamic factors could either inhibit (PIF) or stimulate (PRF) or both depending on the stage of the reproductive cycle. The most potent PRF identified in birds so far, is vasoactive intestinal peptide (VIP). This hypothalamic agent was demonstrated to stimulate PRL release in chicken and turkey pituitary cell cultures (Macnamee *et al* 1986; Proudman and Opel 1988; Chaiseha *et al* 1998b). Immunoneutralization of endogenous VIP blocks PRL release and, thus, prevents hyperprolactinemia and broodiness (El Halawani *et al* 1997).

2.4.1 Vasoactive Intestinal Peptide (VIP)

The hypothalamic factor VIP was originally isolated and identified from gastrointestinal tract extracts (Said and Mutt 1970). This hypothalamic secretagogue is a 28 amino acid linear polypeptide belonging to the secretin-glucagon-VIP superfamily. In turkeys, hypothalamic VIP content appears to be in synchrony with PRL mRNA abundance (Mauro *et al* 1989; Talbot *et al* 1991; Tong *et al* 1998; Chaiseha *et al* 1998a) and plasma level of PRL during the various stages in turkey reproductive cycle (Youngren *et al* 1996).

The number of detectable VIP immunoreactive neurons in the median eminence and medial basal hypothalamus appears to be higher in incubating than in

laying chickens (Sharp *et al* 1989b). These VIP immunoreactive cells were reported to be in close proximity to the hypophysial portal blood vessels (El Halawani *et al* 1997). Therefore, a higher concentration of VIP could be detected in the hypophysial portal blood than in the general circulation (Youngren *et al* 1996). The pulsatile release of VIP into hypophysial portal blood (Chaiseha *et al* 1998b) reflects the status of PRL in the various stages of the turkey reproductive cycle.

The detection of VIP-specific receptors in the turkey anterior pituitary gland provides additional physical evidence for the link between VIP and PRL release (Rozenboim and El Halawani 1993). Accordingly, the concentration of these VIP-specific receptors in pituitary cell membrane varies in concert with pituitary and plasma PRL level during the various stages of the turkey reproductive cycle (El Halawani *et al* 1990).

2.4.2 Dopamine (DA)

Dopamine (DA), a catecholamine and neurotransmitter in the hypothalamus, generally known as a (PIF) in mammals was initially shown to induce PRL release in birds. Subsequent investigations in birds revealed a dual DA action on PRL release: inhibitory during the egg-laying phase and stimulatory during the incubation stage (Youngren *et al* 1995). The mechanism(s) of the suppressive action of DA on PRL release is not understood. The manifestation of an inhibitory DA control on PRL release at the transcriptional level decreases PRL mRNA (Maurer 1980). Dopamine could also exert an inhibitory action at the level of the anterior pituitary gland through D2 DA receptor (Youngren *et al* 1998).

The inhibitory effect of DA on PRL release appears to decline during the transition from egg laying to the incubation phase of the turkey reproductive cycle. A substantial decline in the number of DA binding sites observed in incubating chicken may be attributed to the rise in plasma PRL level at the onset of the incubation period (Macnamee and Sharp 1989).

2.4.3 Serotonin (5-HT)

Serotonin (5-HT) another secretagogue from the hypothalamus, is the precursor of melatonin a hormone involved in various physiological changes in response to photoperiods in vertebrates. This hypothalamic factor has been demonstrated to mediate the photoperiodic response involving PRL secretion during the various phases of the avian reproductive cycle (Youngren *et al* 1996; Pitts *et al* 1996). The 5-HT factor appears to promote a stimulatory PRL response via the dopaminergic and VIPnergic systems (Pitts *et al* 1996).

2.4.4 Thyrotropin releasing hormone (TRH)

In mammals, TRH has a stimulatory effect on the release of PRL. In birds, early *in vitro* studies suggest that TRH could stimulate PRL release from the pituitary gland through the cAMP pathway (Hall *et al* 1985). Accordingly, the effect of TRH on prolactin release was investigated in juvenile and adult turkeys (Proudman 1984). The results indicate that in young turkeys, TRH induce a transient increase in plasma PRL level (Proudman 1984; Fehrer *et al* 1985a,b) but not in adult birds and *in vitro* pituitary cell cultures (Proudman 1984; Fehrer *et al* 1985a,b; Saeed and El Halawani 1986).

2.5 Prolactin gene structure and regulation of its expression in domestic galliformes

The gene encoding PRL has been mapped, cloned and characterised in several vertebrates. In chicken and quail, the PRL gene has been cytologenetically mapped into chromosome 2;p1.1 (Suzuki *et al* 1999b). Cloning and characterisation of the chicken PRL gene revealed that the cDNA nucleotide sequence encodes for 229 amino acid residues (Hanks *et al* 1989; Watahiki *et al* 1989). Thirty of the amino acids make up the signal peptide while the mature hormone is composed of the remaining 199 amino acid residues. Hanks *et al* (1989) demonstrated that there is a high homology in the PRL gene of galliforme species.

2.5.1 The turkey prolactin gene structure

The gene encoding the turkey PRL (tPRL) was initially cloned and characterised based on its amino acid sequence by Karatzas *et al* (1990) who showed that tPRL cDNA is 90% homologous to cPRL cDNA (Hanks *et al* 1989). Similar to the chicken the cDNA encodes a 229 amino acid residue prehormone, 199 of which constitute the mature polypeptide. The nucleotide sequence (~10kb) comprising five exons and four introns including ~2.0kb promoter region of the structural gene encoding for tPRL was reported by Kurima *et al* (1995).

2.5.2 The regulation of prolactin gene expression

In the PRL gene, as in other eukaryotic protein-coding genes, transcription is regulated by its 5' flanking region known as the promoter. This regulatory region is usually but not exclusively located at the 5'flanking region. The promoter contains short DNA sequences that serve as recognition and/or binding sites for proteins essential for the initiation, maintenance and efficiency of protein-coding gene

transcription (McKnight and Kingsbury 1982; Cooper 1992). The presence of an evolutionary conserved TATAA box between 20 to 30 nucleotide bases upstream of exon 1 is essential for site-specific initiation of transcription and has become the ultimate marker for the accurate location of open reading frames (ORFs) in "unknown" DNA segments.

In addition to the universal TATAA homology, a class of regulatory short DNA sequence has been detected both upstream and downstream of the TATAA box. These regulatory sequences appear to direct the efficiency and maintenance of transcription by acting as transcription binding sites (Cooper 1992). There are several hundred of known (and still unknown) proteins that recognise and bind to consensus sequences in the eukaryotic gene promoter region (TRANSFAC, Heinemeyer *et al* 1998).

These DNA motifs may not be as highly conserved as the TATAA box and may have species-dependent variations (McKnight and Kingsbury 1982) but single or multiple base mutation in the consensus sequence disrupt the normal transcription processes (Cooper 1992). Thus, gene products translated from genes with promoter mutation(s) could influence its biological role even though the promoter is not a coding region. Cooper (1992) reviewed the various human pathological lesions that resulted from mutations in the gene promoter region.

2.6 Transcription factors and the prolactin gene

The regulation of PRL gene expression in vertebrates is attributed to several DNA motifs present in the 5'flanking region including the TATA box and other

transcription factor binding sites. Studies of mammalian PRL gene structure, ontogeny and expression identified several transcription factor-binding motifs in the PRL promoter. These short DNA motifs include the Pit1-binding consensus sequence ($^{TT}/_{AA}$TATNCAT in rat, Ingraham *et al* 1988; Nelson *et al* 1988), cyclic AMP binding motif sometimes known as cAMP response element or CRE (TGA$^{AT}/_{TA}$A in rat, Gutierrez-Hartmann *et al* 1987; Keech and Gutierrez-Hartmann 1989; Day and Maurer 1989) and oestrogen response element, ERE (AGGTCAN$_3$TGACCT, Faisst and Meyer 1992). In addition, the DNA-binding motif of AP1 (TGA$^{G}/_{C}$T$^{C}/_{A}$A, Faisst and Meyer 1992) and/or AP2 (CCC$^{A}/_{C}$ N$^{G}/_{C}$ $^{G}/_{C}$ $^{G}/_{C}$, Faisst and Meyer 1992) were also observed (Iverson *et al* 1990).

In birds, the putative Pit1-binding consensus sequence similar to the P2 and P3 in mammals were detected upstream of the PRL promoter of galliforme species (Kurima *et al* 1995; Ohkubo *et al* personal communication). A recent study demonstrated the binding of PRL promoter elements to chicken pituitary nuclear extracts and Pit1 expression vector transfected cells (Ohkubo *et al* personal communication). This indicates that functional Pit1 binding motifs are present in the avian PRL gene promoter. In addition to Pit1, short AP1-like and CRE-like DNA-binding motifs but not ERE were also observed (Ohkubo *et al* personal communication).

2.6.1 Pit/GHF1

Pit1, also known as GHF1, is a homeodomain-containing protein observed to bind specific DNA sequences and activate cell-specific elements on both PRL and

GH gene in mammals (Fox *et al* 1990; Davis 1990; Nelson *et al* 1988). The Pit1 protein binds to the $^{A}/_{T}{}^{A}/_{T}$TATNCAT consensus sequence of the rat PRL promoter either as a monomer or as a dimer and forms a helix-turn-helix protein-DNA interaction (Ingraham *et al* 1988). This configuration and interaction could then activate pituitary-specific gene transcription (Jacobson *et al* 1997; Kato and Kato 1999). In addition, it was also shown to play a role in cell differentiation and proliferation during pituitary gland development (Castrillo *et al* 1991).

Studies on the rat identified four Pit1 binding sites (P1, P2, P3 and P4) in the proximal promoter region (position –190 to –38) of the PRL gene (Day and Maurer 1989; Iversion *et al* 1990). These studies also show that changes in nucleotide sequence within these response elements altered PRL gene transcription. The regulation of PRL gene expression in birds is likewise influenced by the pituitary specific factor Pit1. Ohkubo *et al* (1996) consequently determined the nucleotide sequence $^{A}/_{T}$NCTNCAT as the consensus sequence for the avian Pit1/GHF1 binding site. In chicken, a total of 6 Pit1 binding sites were found, three are located at the proximal region (–128 to –67) and three at the distal region (–1314 to –1128). The proximal promoter region of PRL gene containing putative Pit1 binding sites appears to be highly conserved in mammals and birds (Ohkubo *et al*, personal communication).

Finally, Pit1 has also been demonstrated to interact with other nuclear factors and/or cofactors such as CREB and Est forming a protein-protein complex to specifically promote PRL gene expression (Howard and Maurer 1995). In a recent study, Pit1 was demonstrated to bind to c-*fos* serum response element (SRE)

supporting its role in somato-lactotroph cell proliferation (Gaiddon *et al* 1999). This transcription factor was shown to interact with the zinc-finger protein family (GATA) particularly GATA2, and plays a role in positional determination of pituitary cell types long before the appearance of terminally differentiated cells appear (Dasen *et al* 1999).

2.6.2 cAMP response element (CRE)

Cyclic adenosine 3',5'-monophosphate (cAMP) is a second messenger that carries signals from the cell surface to intracellular proteins via protein kinase A (PKA) which in turn, mediates the phosphorylation of CREB at Ser133 (Radhakrishnan *et al* 1997). The activated CREB regulates PRL gene transcription through binding short DNA sequences ($TGA^{AT}/_{TA}A$ or CRE) in the rat PRL promoter in pituitary (Yan *et al* 1994; Keech *et al* 1992; 1989) and nonpituitary cell lines (Liang *et al* 1992).

The sequence analysis of the 2.6kb cPRL promoter by Ohkubo *et al* (personal communication) have shown that the cPRL upstream region did not contain the CRE DNA motif ($TGA^{AT}/_{TA}A$). However, the group reported a CRE-like sequence (TGACGTGC) was detected 2bp downstream of the TATA box and suggested that this CRE-like motif may contribute to the regulation of the cPRL gene.

2.6.3 The AP1 family of transcription factors

The transcription factor AP1 is a member of the leucine zipper factors whose DNA-binding region is followed by repeated leucine residues. The consensus binding motif for AP1 is $TGA^{G}/_{C}T^{C}/_{A}A$ (Faisst and Meyer 1992). The binding of AP1 to a

eukaryotic gene promoter manifests either a positive or a negative control over its transcription. The potency of AP1 control of transcription activity is enhanced by its interaction with Fos.

2.7 The development of DNA molecular markers to aid future breeding programs

The influence of avian PRL in the maintenance and/or initiation of incubation behaviour are invariably supported by the changes in the hypothalamo-hypophysial-ovarian axis associated with broodiness. Current management and selective breeding strategies have undoubtedly improved production rate of economically valuable traits. The cost of production management required in preventing broodiness in turkey however, influence its commercial viability. Alternative cost effective strategies for the accurate identification and selective breeding of non-broody hens without affecting other commercial traits are necessary to improve the rate of production.

The development of DNA markers for trait selection may provide alternative cost effective methods in animal breeding and production (Hillel 1994; Hillel *et al* 1993). The DNA-based markers are nucleotide sequences in several physical regions or loci and whose inheritance can be monitored. Sequence variations detected in candidate genes associated with a trait could be employed as molecular markers to select for or against such a trait. To this effect, the identification of PRL gene promoter variants associated with broodiness could provide insights on the regulatory mechanism(s) of tPRL gene transcription as well as potential DNA markers for future breeding programs.

2.8 Statement of the Objective

This study is conducted to detect and identify sequence variations in the tPRL promoter region by PCR-SSCP analysis. The polymorphisms detected will be characterised and the genotypic frequency within the population studied was be determined. The effect(s) of these genetic variants on the levels of plasma PRL will be evaluated.

CHAPTER III. MATERIALS AND METHOD

3.1 Blood sampling and the identification of the turkey physiological state

Blood samples (*5ml*) were obtained from 62 female British United Turkey (BUT) large white turkeys from the 22nd to the 56th week of age by brachial puncture into heparized tubes. The blood samples were collected every three weeks from 22 to 28 weeks and every two weeks from the 30th to the 42nd week. Subsequently blood was collected every three weeks from the 45th to the 56th week. The sampling schedule corresponded to the various stages of the turkey reproductive cycle. Following centrifugation, the plasma and blood cells were frozen (–20°C) separately for later analysis.

Egg production and expression of incubation behaviour were recorded daily following the onset of egg laying. Nest boxes were checked four times daily (0900, 1100, 1600 and 1800h) throughout the experimental period. The hens were considered to be incubating when detected in a nest box on a minimum of three out of four checks during three consecutive days. Of the 62 birds, 18 were incubating and 44 were not incubating.

3.2 The plasma prolactin radioimmunoassay

The blood plasma obtained from 62 was assayed to determine the concentration of plasma PRL during a 35-week turkey reproductive cycle. The concentration of plasma PRL was determined in triplicate using the similar radioimmunoassay protocol described by Guemene *et al* (1994). The similar

radioimmunoassay protocol employs a recombinant turkey PRL (rtPRL, Karatzas *et al* 1993). All samples were assessed in a single assay to avoid interassay variation. The intra-assay coefficient of variation was 4.5%.

3.3 Extraction of genomic DNA

The frozen blood cells were thawed in ice and aliquots of 10μl were placed into 1.5*m*l Eppendorf tubes containing 500μl 0.5mM sodium acetate (pH7). A 25μl aliquot of 20% sodium dodecyl sulfate (SDS) was added into each tube containing the blood cells and sodium acetate mix to lyse the cell membrane and other cellular components, thus exposing the DNA. The DNA was then separated from other cellular components by extraction using an equal volume of 25:24:1 phenol:choloform:isoamyl solution. After thorough agitation, the mix was centrifuged at 10,000 *rpm* for 10min. The lower phase, containing phenol:chloroform:isoamyl and extracted proteins, was discarded while the upper aqueous phase was kept for a second extraction. Genomic DNA recovery was performed by 100% cold ethanol precipitation and the recovered DNA was dissolved in 5T.1E (5mM Tris-HCl pH 7.4, 0.1mM EDTA) buffer or water. The DNA concentration was estimated by its optical density at 260nm (OD_{260}) using a spectrophotometer (Sambrook *et al* 1989).

3.4 The polymerase chain reaction strategy

Eight primer pairs (A to H) were designed based on sequence of the 5'flanking region of tPRL gene reported by Kurima *et al* (1995) covering 2.162kb of

the tPRL promoter (Table 3.4). An additional primer pair (A_d) based on similar sequences in the cPRL (Ohkubo *et al* personal communication), was designed to extend the tPRL sequence at the 5'end. The primer pairs were intended to amplify nine overlapping fragments, comprising approximately 2.4kb of the tPRL gene 5'flanking region (Fig 3.4).

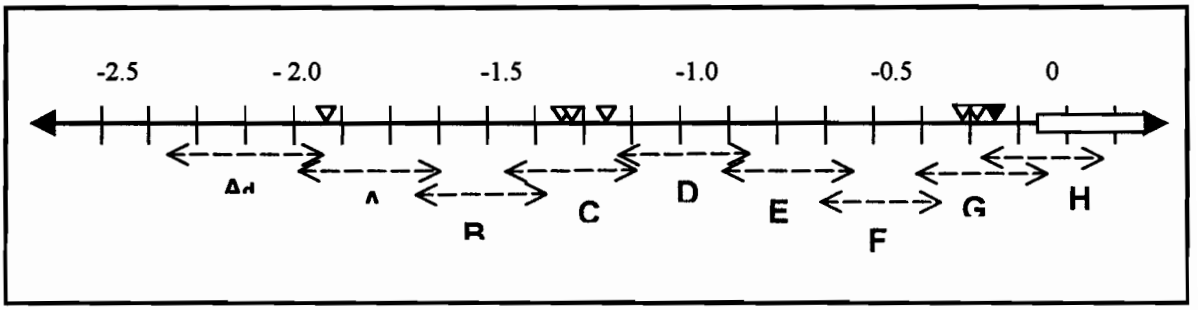


Figure 3.4 The 2.5kb tPRL promoter region. The expected location of fragments Ad to H are indicated by a <----> . The locations of the putative Pit1 binding consensus sequences are indicated by ∇ and the TATA box by a ▼ . Exon 1 is represented by □.

Fragment	Primer	Primer Sequence	Location	Size (bp)
Ad	Ad_F	AAGAGCACAAACCATGTATG	-2324 to -1954	370
	Ad_R	AGGACTTTGGACTCAGTGAT		
A	A_F	TCATAGGGTTTGGAAGGCAC	-1963 to -1637	327
	A_R	GAGAAGATTGAGTGGATCTC		
B	B_F	CCTGGCCCCATTCTCATCCC	-1699 to -1370	331
	B_R	AGCAGTGTGCCCTTGCGGCC		
C	C_F	AGCCCACGGTCAACCTGTTG	-1369 to -1050	331
	C_R	GTTCTGTACATGTGGAACAGG		
D	D_F	ACAGTTACGAAATAATGGGAG	-1110 to -810	301
	D_R	GTCGTAATCAGTGGGAATCTG		
E	E_F	GATCAGGGAATCAGATTCCAC	-839 to -485	354
	E_R	ATGGAGGCTTCTGGAAAGAC		
F	F_F	GGAGACAAACACACACTACG	-538 to -240	319
	F_R	GAGTATGGCTGGATGAAGAG		
G	G_F	CATTTGCAACTAATTCAGTGC	-239 to 88	362
	G_R	GTCTTACCTTTCAATGAAGCC		
H	H_F	GACATGCAGAAAGTAAGAGC	-19 to 169	188
	H_R	TCATAGGGTTTGGAAGGCAC		

Table 3.4. The primer sequence, location and size of expected fragment. The forward primer and the reverse primers are designated by an F and R subscripts respectively.

3.4.1 The PCR amplification of fragments A_d to H

Each primer pair was used to amplify specific yet overlapping regions of the tPRL promoter (Fig 3.4). For each PCR reaction, a reaction mix containing 50*ng* of genomic DNA, 10*n*M of each primer, 2.5μl 10x *Thermus thermophilus* (*Tth*) DNA polymerase enzyme buffer (10*mM* Tris-HCl pH 9.0, 15*mM* $MgCl_2$ and 500*mM* KCl), 50μM of each dNTP and water up to a 24μl total volume was prepared prior to thermal cycling. At the same time, an enzyme mix containing 0.625 units of *Tth* polymerase and water up to 1μl in total volume was also prepared for each reaction and stored on ice. The microcentrifuge tubes containing the reaction mix were subsequently placed into a DNA Thermal Cycler 480 (Perkin Elmer Cetus Corp, New Jersey, USA). The initial denaturation executed at 95°C for 5min was followed by the addition of 1μl enzyme solution to each PCR reaction mix bringing the total volume of each reaction solution to 25μl. The addition of the DNA polymerase after the initial denaturation of the genomic DNA, or hot start, increases the specificity of primer annealing to the target sites.

The thermal cycler was programmed for 35 cycles, the first 5 of which consisted of 94°C for 1min denaturation, 65°C for 1min and 30sec annealing and 72°C for 2min extension time. The next five cycles were similar to the previous settings except for the lowering of the annealing temperature to 62°C. This was followed by 25 cycles wherein the annealing temperature was further reduced to 60°C. After the completion of 35 cycles, approximately 10μl of the PCR amplified fragments were transferred to clean tubes containing 2μl of 5x gel loading buffer

(15% Ficoll in water, 0.25% bromophenol blue and 0.25% xylene cyanol). Each PCR product and a ϕx174 *HincII* DNA digest molecular weight marker were separated in a 2.5% agarose gel with a at 100v for approximately an hour. The gel was subsequently stained with ethidium bromide. The results were visualised in a UV light box and photographed using Kodak Digital Science DC210 Zoom Camera. The digital image was then processed by the 1D Image Analysis software (Kodak Canada Inc., Toronto, Ontario, Canada).

3.5 The single-strand conformation polymorphism (SSCP) analysis

The single-strand conformation polymorphism (SSCP) originally described by Orita *et al* (1989), was based on the principle that base changes in single-stranded DNA could modify the DNA conformation and could create a mobility shift as the strands migrate on a nondenaturating polyacrylamide gel (PAG) during electrophoresis. The technique has been demonstrated to be sensitive in detecting single base substitution, insertions and deletions particularly in 300 to 400bp fragments (Hongyo *et al* 1993). The sensitivity for the detection base changes using SSCP is influenced by temperature and other electrophoretic conditions such as the degree of cross-linking in the gel and the voltage used (Fan *et al* 1993).

A mix of 1μl PCR product and 15μl of gel loading buffer (95% formamide, 20*mM* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) were initially denatured at 98°C and separated in three preliminary SSCP conditions. Trial SSCP runs tested several gel concentrations including 8%, 8% +5% glycerol, and 15% polyacrylamide:bisacrylamide (PAG) at 49:1 percent crosslinking and electrophoretic

Fragment	Gel concentration	5% Glycerol	Migration speed (v/cm)	Duration (hours)	Temperature (°C)
A	15%	-	8.5v/cm	20	Room
	12%	-	8.5v/cm	7	Room
	9%	-	8.5v/cm	7	Room
	8%	+	8.5v/cm	10	Room
	8%	-	8.5v/cm	7	Room
B	15%	-	8.5v/cm	12	Room
	9%	-	**8.5v/cm**	**7**	**Room**
	8%	+	8.5v/cm	10	Room
C	15%	-	8.5v/cm	10	Room
	9%	-	**8.5v/cm**	**7**	**Room**
	8%	+	8.5v/cm	10	Room
	8%	-	8.5v/cm	7	Room
D	20%	-	8.5v/cm	24	Room
	15%	-	8.5v/cm	17	Room
	12%	-	8.5v/cm	15	Room
	12%	-	8.5v/cm	10	25
	9%	-	8.5v/cm	8	Room
	8%	+	8.5v/cm	10	Room
	8%	-	8.5v/cm	8	Room
E	20%	-	8.5v/cm	20	Room
	15%	-	8.5v/cm	16	Room
	12%	-	8.5v/cm	10	Room
	9%	+	8.5v/cm	10	Room
	8%	-	8.5v/cm	7	Room
	8%	+	8.5v/cm	10	Room
E digested	15%	-	8.5v/cm	8	Room
	9%	-	**8.5v/cm**	**6**	**Room**
	8%	+	8.5v/cm	8	Room
F	20%	-	8.5v/cm	20	Room
	15%	-	8.5v/cm	16	Room
	12%	-	8.5v/cm	15	Room
	8%	+	**8.5v/cm**	**10**	**Room**
	8%	-	8.5v/cm	8	Room
G	20%	-	8.5v/cm	24	Room
	15%	+	8.5v/cm	30	Room
	15%	-	8.5v/cm	12	28
	15%	-	**8.5v/cm**	**17**	**Room**
	12%	-	8.5v/cm	10	Room
	8%	+	8.5v/cm	7	Room
H	20%	-	8.5v/cm	20	Room
	15%	-	8.5v/cm	5	Room
	8%	+	8.5v/cm	7	Room
A_d	15%	-	8.5v/cm	17	Room
	8%	+	8.5v/cm	10	Room
	8%	-	8.5v/cm	7	Room

Table 3.4. Optimization of SSCP conditions. The PAG cross-linking is 49:1 acrylamide:bisacrylamide and the optimized condition utilized for genotyping are in bold letters. Room temperature is approximately 21°C.

conditions at 8.5v/cm for approximately 8, 12, and 17h respectively (Table 3.5). The gel electrophoresis was performed on a Mini Protean II apparatus (BioRad Laboratories, Hercules, California, USA) containing 1x TBE buffer (89*m*M Tris base, 89*m*M Boric acid and 2*m*M EDTA pH 8.0) and silver stained. Silver staining was accomplished by soaking the PAG in glutaraldehyde followed by six 10min washes in water. The PAG was subsequently placed in solution containing 0.2% sodium hydroxide (NaOH), 1% ammonium hydroxide (NH_4OH) and 0.2% silver nitrate ($AgNO_3$) for 10min and rinsed with water twice. The gel was then transferred to another solution containing 0.005% formaldehyde and 0.5% citric acid. After a few minutes of gentle shaking the DNA could be visualised as dark bands in the gel. The results were visualised on a light box and subsequently photographed using a Kodak Digital Science DC210 Zoom Camera. The digital image was then processed by the 1D Image Analysis software (Kodak Canada Inc., Toronto, Ontario, Canada).

The SSCP trials were necessary to determine the optimal condition for the detection of sequence variation(s) in the single-stranded DNA. The electrophoretic conditions tested and the optimal SSCP conditions determined for each PCR amplified fragment are listed in Table 3.5.

3.6 Purification of the PCR products

About 25μl of PCR product from two samples representing each fragment (fragments A_d to H) were separated in a 1% agarose gel at 100v for 10min. The gel was subsequently stained with ethidium bromide, visualized under UV light and the DNA from each lane was excised from the gel. Each gel piece containing one DNA

fragment was minced to approximately $1mm^3$ pieces and placed into labeled, new microcentrifuge tubes. The DNA fragment was subsequently eluted from the gel and purified with Qiagen columns (Qiagen PCR Purification Kit) using the protocol recommended by the manufacturer (Qiagen, Missisauga, Ontario, Canada). The purified DNA was subsequently used for sequence analysis.

3.7 Cloning of the G fragment

Fresh PCR products from heterozygous individuals were inserted into a plasmid vector (pCR TOPO) provided by the TopoTA cloning kit (Invitrogen, Carlsbad, California, USA). The cloning procedure was carried out according the protocol provided by the manufacturer. In brief, 2μl of fresh PCR products and 1μl of pCR TOPO vector were incubated at room temperature for 5min, centrifuged briefly and stored in ice. At the same time, 0.5M β-mercaptoethanol was added into several vials of competent cells prior to transformation. Two microliter of each sample mix was added to a vial containing competent cells and incubated in ice for 30min. The cells were subsequently heat shocked at 42°C for 30sec and immediately transferred to ice. After incubating at room temperature for 2min, 250 μl of SOC medium (2% bacto-tryptone, 0.5% yeast extract, 10*m*M NaCl, 2.5*m*M KCl, 10*m*M $MgCl_2$, 10*m*M $MgSO_4$, and 20M glucose, pH6.7 to 7.0) was added to each vial and placed in a shaker water bath at 30°C for 30min.

Several agar (1.5%) plates containing LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl, pH7.0) and 50μg/ml ampicillin were prepared. About 40μl of

40*m*g/ml X-Gal and 40μl of 100*m*M IPTG were coated on the surface of the agar and incubated at 37°C prior to use. Approximately 50 to100μl of the transformed cells were spread on the warm plates and incubated at 37°C overnight. White colonies were selected and transferred to vials containing LB medium with 50μg /ml ampicillin and incubated for another 8h.

The plasmids containing the insert were isolated from the cells using the mini-prep protocol described by Sambrook *et al* (1989). The insert was extracted from the bacterial plasmid by *EcoRI* digestion and amplified using the PCR protocol described earlier.

3.8 Direct sequencing of fragments A_d to H.

The purified PCR amplified fragments A_d, A, B, C, D, E, G and H were directly sequenced using the procedure provided in the ABI Big Dye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, California, USA). The reaction mix for each sequencing reaction contained 50*n*g of template DNA, 4μl of the terminator ready reaction mix provided in the kit, 3.2μM either forward or reverse primer and deionized water to make up to 10μl volume. The tubes containing the reaction mix were placed in the thermocycler (DNA Thermal Cycler 480) which was programmed for 25 cycles. Each step cycle has a denaturation temperature of 96μl for 10sec, 50°C annealing temperature for 10sec, and 60°C extension temperature for 4min. The sequencing extensions were purified and precipitated using 75% isopropanol at room temperature for approximately 24h. The precipitate was

subsequently pelleted by centrifugation at maximum speed for 20min and the DNA pellet obtained was redissolved in 5μl of loading buffer (5:1 deionized formamide: 25*m*M EDTA pH8.0 and 50*mg/m*l blue dextran). Approximately 2μl of each sample was loaded in an ABI Prism 377 DNA Sequencer (PE Biosystems, Foster City, California, USA). The processing and interpretation of the fluorescence signal collected in a Macintosh computer were accomplished using a Data Collection Software and Sequence Analysis Software provided by the ABI Prism 377 DNA Sequence manufacturer.

Fragment F was the only fragment sequenced manually using the Cycle Sequencing Kit (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). The protocol recommended by the manufacturer required the end labeling of either forward or reverse primer with 5μCi δ^{32}P (ICN Biomedicals, Aurora, Ohio, USA) using T4 polynucleotide kinase (PNK) provided in the kit. The labeled primer was added to a PCR mix containing 25μM of the dNTP mix including a 7-deaza dGTP, *Thermus aquaticus* (*Taq*) DNA polymerase buffer (100*m*M Tris-HCl pH 9.0, 15*m*M $MgCl_2$ and 500*m*M KCl) and 0.5 units of *Taq* DNA polymerase provided in the Cycle Sequencing kit. Aliquots of 10μl were transferred to new tubes labeled A, C, G, and T which contain the dideoxynucleotide termination solution ddATP, ddCTP, ddGTP, and ddTTP respectively. A 10*n*g template DNA was subsequently added to each tube containing radiolabeled PCR reaction mix and the tubes were transferred to a thermal cycler programmed for 25 cycles. Each step cycle was at 95°C denaturation for 30sec, 55°C annealing for 36sec and 72°C extension for 84 sec. A stop solution provided with the kit was added to each tube at the end of the last step cycle.

A 6% PAG sequencing gel at 29:1 percent acrylamide:bisacrylamide crosslinking containing 8.5M urea and 1x TBE buffer (89*m*M Tris base, 89*m*M Boric acid and 2*m*M EDTA pH 8.0) was prepared in a Vertical DNA Electrophoresis Sequencing Cell (BioRad Laboratories, Hercules, California, USA) apparatus as the PCR was on the step cycling program. Prior to loading the samples, the gel was run at 50watts (W) for 30min.

After the PCR cycling program was completed, aliquots of 5μl from each A, C, G, and T samples were transferred to a water bath with boiling water for 2min. The tubes were subsequently transferred to ice prior to loading. About 4μl from each sample was loaded in a specific order in the previously prepared PAG. The electrophoretic condition was at 65W constant power for two to four hours. Upon completion of the gel electrophoresis, the gel was removed from the glass plates with a Whatman paper, covered with Saran Wrap and dried in a BioRad Gel Dryer model 583 (BioRad, Hercules, California, USA). The dried PAG was exposed to a Kodak X-ray film (Kodak Canada Inc., Toronto, Ontario, Canada) for 72h and subsequently developed. The results were photographed using a Kodak Digital Science DC210 Zoom Camera and processed by 1D Image Analysis software (Kodak Canada Inc., Toronto, Ontario, Canada).

3.9 Restriction fragment length polymorphism of the E fragment

A polymorphic *HindIII* restriction site (A↓AGCTT) was detected in fragment E following sequencing of this fragment. Accordingly, the genotyping of fragment E was accomplished incubating 5*n*g of PCR amplified DNA with one unit of *HindIII*

restriction enzyme overnight at 37°C. The digested products were separated in a 1% agarose at 100v for 10min. The DNA was stained with ethidium bromide and visualised using a light box. The gel was subsequently photographed using a Kodak Digital Science DC210 Zoom Camera. The digital image was then processed by the 1D Image Analysis software (Kodak Canada Inc., Toronto, Ontario, Canada).

3.10 Statistical Analysis

Analysis of variance (ANOVA) was performed to test the interaction between genotypes and the plasma PRL concentration during the various stages of the turkey reproductive cycle. Using the two-factor ANOVA the effect of genotypes on the plasma PRL concentration was dependently calculated for fragments B, C, E, F and G genotypes. The PRL response to genotype were also assessed in the incubating and nonincubating subgroups.

3.11 Transcription factor search

A search for short DNA motifs in the tPRL promoter nucleotide sequence similar to known transcription factor binding sites was performed using Gene Tool Software. A search was also executed in the TRANSFAC database available in the world wide web and only binding sites 90% similar to the tPRL promoter short DNA sequences were included. The search results were screened for putative transcription factor-binding sites for transcription factors that are known to bind to PRL promoters. The search included potential transcription binding sites for transcription factors

determined to influence PRL gene expression and/or pituitary cell differentiation and proliferation.

CHAPTER IV. RESULTS AND DISCUSSION

4.1 PCR amplification of fragments A_d to H

The primers which were based on the reported tPRL promoter (U05953, GenBank accession number U05953) amplified a total of eight fragments covering the 2.162kb of the tPRL 5'flanking region (Fig.3.4). The eight fragments subsequently obtained were designated as fragments A to H (Fig 4.1a and 4.1b), A being the most distal and H the most proximal. The band amplified from each primer pair was within the expected range of 188 to 370bp, indicating that the PCR has specifically amplified the target DNA segment (Table 3.4).

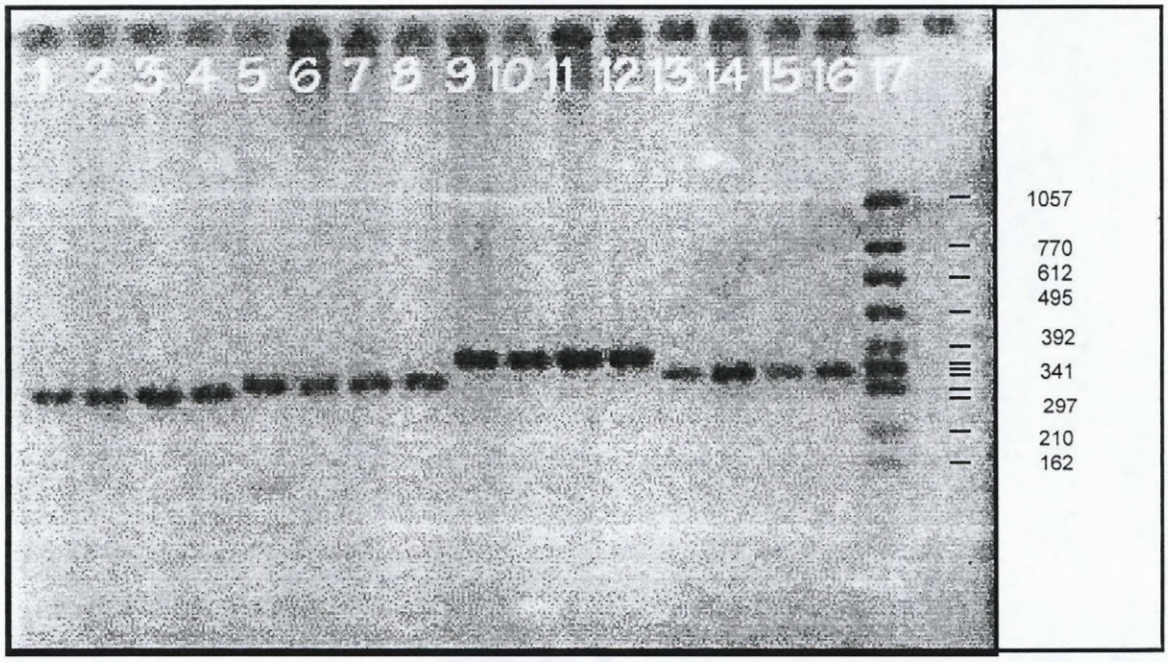


Figure 4.1a. PCR amplified tPRL promoter fragments A to D. The PCR amplified fragments A, B, C, D, and a ϕx174 *Hinc II* DNA digest molecular weight marker were separated in a 2.5% agarose gel at 100v for 1h. Lanes 1 to 4 are fragment D, lanes 5 to 8 are fragment C, lanes 9 to 12 are fragment B and lanes 13 to 16 are fragment A samples. The ϕx174 *Hinc II* DNA digest molecular weight marker is in lane 17.

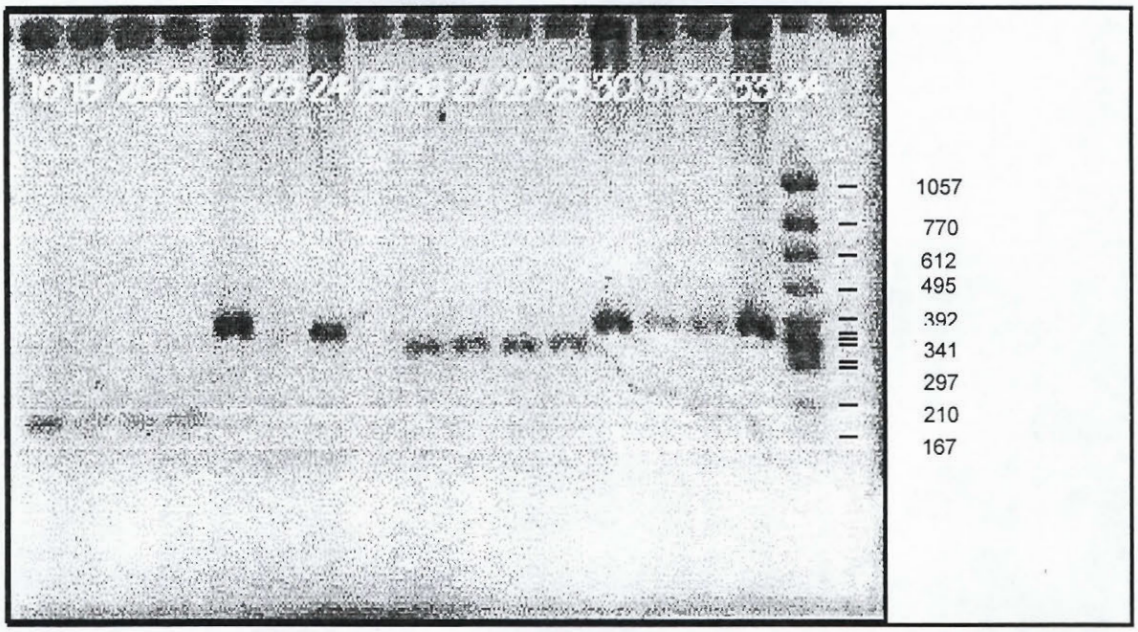


Figure 4.1b. PCR amplified tPRL promoter fragments E to H. The PCR amplified fragments E, F, G, H, and a ϕx174 *Hinc II* DNA digest molecular weight marker were separated in a 2.5% agarose gel at 100v for 1h. Lanes 18 to 21 are fragment H, lanes 22 and 24 are fragment G, lanes 26 to 29 are fragment F and lanes 30 to 33 are fragment E samples. The ϕx174 *Hinc II* DNA digest molecular weight marker is in lane 34.

Recently, Ohkubo and colleagues (personal communication) cloned and characterised the chPRL gene including approximately 2.6kb of its promoter region. The 2.2kb of the chPRL promoter were about 90% identical to the reported tPRL promoter nucleotide sequence (U05953). The high sequence similarity indicated the possibility of extending the tPRL promoter sequence using PCR primers based on the promoter nucleotide sequence in the two birds. A new primer pair was subsequently designed to extend the known tPRL promoter sequence by 265bp. The reverse primer (A_{dR}) was anchored at –1973 to –1954 of the tPRL promoter while the forward primer (A_{dF}) was designed based on the nucleotide sequence at –2305 to –2324 of the chPRL promoter sequence (Table 3.4). The new primer pair has a 65bp overlap with

the most distal of the tPRL 5'flanking region (Fragment A) to increase the chance of a successful and accurate amplification of the target fragment. The primer pair A_{dF} and A_{dR} amplified a 370bp fragment distal to fragment A subsequently designated as fragment A_d. The successful amplification of the turkey fragment A_d using a combination of the chicken and turkey primer sequences support high percentage of similarity in the PRL promoter nucleotide sequence in both birds.

4.2 The PCR-SSCP analysis and characterisation of Fragment A_d

The SSCP analysis of the 370bp fragment A_d using the three preliminary conditions (Table 3.5) revealed no variation in the migration pattern of the single stranded DNA. This result suggested that genetic variants in this region may be present in the population but were not detected in the SSCP analysis of 62 individuals. Therefore it is likely that allelic variants at this locus are relatively rare. However, we do not preclude the possibility that the electrophoretic conditions chosen were not adequate to detect conformational changes.

Only 325bp of the 370bp fragment A_d was clearly determined by sequence analysis. The 96% similarity of the nucleotide sequence obtained to the chPRL promoter indicates that the PCRamplified fragment was the target tPRL promoter sequence (Fig 4.2) The 4% difference between chPRL and tPRL gene promoter within the 325bp sequence includes 4 deletions, a C at –2256, –2252, and –2248 as well as a G at –2138. Two T insertions at –2147 and –2045 and six base substiutions at –2215 (C to T), –2185 (C to G), –1281 (C to T), –2065 (G to A) –2029 (A to C) and –2028 (A to T) were also observed (Figure 4.2)

Since the A_d reverse primer (A_{dR}) has a 65bp overlap in fragment A including the A_F primer site, the A_d base sequence within the overlap was compared to that of the reported tPRL sequence (U05953). The comparison revealed four variations in the nucleotide sequence in the two tPRL promoter. At –1974 a G was replaced by an A, at –1964, an A became a G, at –1955 a T was substituted by a G, and at –1939 to –1936 three bases TGG became CAA (Fig 4.2). The difference in the nucleotide sequence within the 65bp tPRL promoter indicates the presence of species-specific variations in the turkey population.

```
ACCATCCATT CTAGTGTTTT CGGCCTGTCG GCCCTGTTCT TAGTGCCTTG ATCAGATTAT
********** **********  *** *** * ********** ********** **********
ACCATCCATT CTAGTGTTTT ♦GGC♦TGT♦G GCCCTGTTCT TAGTGCCTTG ATCAGATTAC

ACTTATCTCA GCTGGAGGCA ACTACTTCGT CTACCCAGTA AGGCCTCAAT TTCCAAACCA
********** ********** **********  *** ***** ********** **********
ACTTATCTCA GCTGGAGGCA ACTACTTCGT GTACTCAGTA AGGCCTCAAT TTCCAAACCA

GACCCAGGA♦ CTGAACAGAA CAAACTGTGC CTAGAAATCA TCCTAGATCT TAGAAGATAT
*********  ********** ******** * ********** ********** **********
GACCCAGGAT CTGAACAGAA CAAACTGT♦C CTAGAAATCA TCCTAGATCT TAGAAGATAT

AGACTACTTA CTACCAGTTC TGGTTTACTG CCGAAGCATC TCCCTTTATT GAA♦CCCAGT
********** ********** ********** ** ******* ********** *   ******
AGACTACTTA CTACCAGTTC TGGTTTACTG CCAAAGCATC TCCCTTTATT GCTTCCCAGT

GGATGTCTTC CATGCTTTAC CCCCTAAATC ATAGAGTCAT AGGGGTTGGA AGGCACATCC
********** ********** ********** ********** ********** **********
GGATGTCTTC CATGCTTTAC CCCCTAAATC ATAGAGTCAT AGGGGTTGGA AGGCACATCC

CAAAGATCAC TGAGTCCAAA GTCCC
********** ********** *****
CAAAGATCAC TGAGTCCAAA GTCCC
```

Figure 4.2 Sequence analysis of fragment A_d. The upper line consists of chPRL gene upstream sequence from –2276 to –1954 and the second line consists of tPRL gene nucleotide sequence at the same location. Variations in the nucleotide base sequence between chPRL and tPRL are in **bold** letters while deletions and insertions are indicated by a ♦. The underlined nucleotide bases do not match the reported tPRL promoter (U05953) upstream sequence.

4.3 The PCR-SSCP analysis and characterisation of fragment A

The primer pair A forward (A_F) and A reverse (A_R) amplified a 327bp DNA segment located at –1963 to –1637 of the tPRL promoter (Table 3.4). Five preliminary electrophoretic conditions were used to detect alteration in the migration pattern of the single-stranded DNA. A mobility shift in the single-stranded DNA during electrophoresis indicates a difference in conformation due to a variation(s) in the nucleotide sequence. The results obtained from the SSCP analysis of 62 birds showed no difference in the migration pattern of the single-stranded DNA under the five conditions evaluated (Table 3.5). The DNA mobility pattern of one SSCP condition tested is shown in Fig 4.3a.

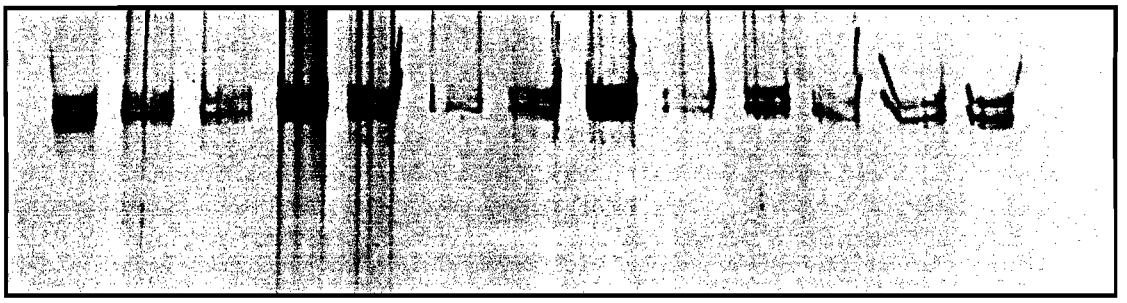


Figure 4.3a The PCR-SSCP analysis of Fragment A. Single-stranded DNA were separated in 8% PAG at 8.75 volts/cm for 7 h. No variation in the migration pattern was observed.

The efficiency of detecting nucleotide base changes based on single strand DNA secondary structure is affected by the location and stability of the secondary structure as well as the electrophoretic conditions (Fan *et al* 1993). Thus, the conditions for analysis are usually tested empirically. In the current study, five different electrophoretic conditions were employed to detect conformational variation(s) within fragment A (Table 3.5). The conditions however, may not have been adequate to detect conformational changes due to other parameters such as

location and stability of the secondary structure. To further verify the SSCP result, two PCR amplified fragment A samples were selected at random for DNA sequencing.

```
TCATAGGG-T TGGAAGGCAC ATCCTGGAGA TCACTGAGTC CAAAGACCTC TGCTAATGCA
******** * ********** ********** ********** ********** **********
TCATAGGGGT TGGAAGGCAC ATCCTGGAGA TCACTGAGTC CAAAGACCTC TGCTAATGCA

GGTTACCTAT AGTAGGTTGT ACAGGAAACT GCCCAGGAAG ATTTTGAGTA TCTCCAGAGG
********** ********** ********** ********** ********** **********
GGTTACCTAT AGTAGGTTGT ACAGGAAACT GCCCAGGAAG ATTTTGAGTA TCTCCAGAGG

ACACTCATCA ATCTCTCTGG GCAGCTTGTT CCACTGCTCT GTCACCCTAA AAGTAAAGTT
********** ********** ********** ********** ********** **********
ACACTCATCA ATCTCTCTGG GCAGCTTGTT CCACTGCTCT GTCACCCTAA AAGTAAAGTT

TTTCCTAATG TTCATATGGA ACTTCCTGTG TTACAGTTTA TCCCCATTGC TCCTTGTTCT
*** ****** ********** ********** ********** ********** **********
TTTTCTAATG TTCATATGGA ACTTCCTGTG TTACAGTTTA TCCCCATTGC TCCTTGTTCT

GTCACTTGGC ACCACCAAAA ACAGCCTGGC CCCATTCTCA TCCCTTTAGA TATCTATAAG
********** ********** * ******** ********** ********** **********
GTCACTTGGC ACCACCAAAA AGAGCCTGGC CCCATTCTCA TCCCTTTAGA TATCTATAAG

CATTGATGAG ATCCACTCAA TCTTCTC
********** ********** *******
CATTGATGAG ATCCACTCAA TCTTCTC
```

Figure 4.3b Sequence analysis of Fragment A. The upper sequence is the published tPRL gene promoter sequence (U05953) from –1963 to –1637; the lower sequence is the variation detected in this study and the nucleotide base changes are in bold letters. The DNA motif for a possible Pit1 binding site (Ohkubo *et al* 1996) is bold and underlined. A putative AP2 binding consensus sequence is in *Italics* and underlined. The putative c-Ets binding DNA motifs are in ***bold Italics***.

The nucleotide sequence obtained from the two samples was 99% identical to tPRL promoter sequence from –1963 to –1637 (U05953). Three changes in the nucleotide base sequence were a G deletion, a C to T and a C to G base substitution at –1955, –1780, and –1702 of the tPRL promoter respectively (Fig 4.3b). The single-base modifications that were observed in both the samples suggest that each turkey

strain may have strain-specific variations in the tPRL gene which could be attributed to the selective breeding process during the development of the BUT strain. Taken together with the SSCP analysis, this may suggest that very few genetic variants are present in this region of the promoter.

Sequence analysis of this fragment (Fig 4.3b) revealed that a short DNA sequence motif within fragment A is identical to the antisense sequence ($ATGNAGN^{T}/_{A}$) of the reported transcription-binding site consensus sequence for teleost and avian Pit 1 ($^{T}/_{A}NCTNCAT$) reported by Ohkubo *et al* (1996). The hypothalamic factor Pit1 (also known as GHF1) is a homeodomain containing pituitary-specific transcription factor capable of directing tissue-specific PRL (Nelson *et al* 1988) and GH (Bodner *et al* 1988) gene transcription and pituitary specific cell proliferation in mammals. Ingraham *et al* (1988) observed that Pit 1 directly initiates PRL gene transcription via *cis*-active elements and induces pituitary specific phenotypes. This role is manifested through Pit1 binding to specific sequences on the distal (Day and Maurer 1989) and proximal regions of PRL and GH gene promoter (Nelson *et al* 1986; 1988). Mutations of the Pit1 binding motif in the distal region reduce Pit1 binding and result in a 20-fold decrease in PRL gene expression (Ingraham *et al* 1988). This result however, appears to counter an earlier finding by Nelson *et al* (1986) indicating a 100-fold increase in PRL gene expression following the removal of the distal (–1831 to –1530) region containing Pit1 binding motifs.

The presence of a putative Pit1-binding motif in fragment A suggests that this DNA segment is comparable to the distal region of the mammalian PRL gene promoter which contain *cis*-acting sequences capable of transcription regulation. The

categorical role of this putative Pit1 binding site located at –1908 to –1901 (Fig 4.3b) is as yet unexplored in turkey. Since the analyses of this region in 62 hens revealed no changes in DNA sequence, the region may be required only for the ontogeny of pituitary cells and/or the formation of an active transcription complex. Further analysis of putative roles is required to verify a functional Pit1/GHF1 binding region.

Subsequent analysis of this region reveals another potential transcription factor binding site at –1700 to –1693 in the tPRL promoter. The nucleotide sequence GCCTGGCC (Fig 4.3b) is similar to the core sequence of the AP2 binding motif ($CCC^{A}/_{C}N^{G}/_{C}{}^{G}/_{C}{}^{G}/_{C}$, Faisst and Meyer 1992) and somewhat similar to CCCCTCCC sequence of the putative AP2 binding motif observed in rat PRL gene promoter (Iverson *et al* 1990). In mammals, transcription factor AP2 plays a role in the ontogeny of cells with ectodermal lineage including the neural tube, and craniofacial and body wall morphogenesis (Creaser *et al* 1996). This *cis*-acting element was demonstrated to mediate cAMP responsiveness and together, CREB and AP2, could form a transcription factor complex which may interact with the 3D Pit1 structure to regulate PRL gene transcription (Iverson *et al* 1990).

In addition, Creaser *et al* (1996) reported a conservation of AP2 *cis*-acting sequence between chicken, mouse and human. This information and the presence of an AP2 binding motif detected in the vicinity of a potential Pit1 binding motif in the tPRL promoter suggest that AP2 and Pit1 interaction may play a role in the PRL gene transcription regulation in birds. However, more research is required to establish the presence of these functional transcription factor binding elements in this region of the tPRL promoter.

Analysis of the nucleotide sequence in fragment A using the transcription factor databank compiled by Heinemeyer *et al* (1998) revealed several short DNA motifs with more than 90% homologies to known transcription binding sites were observed within the fragment. These short DNA sequences include consensus-binding motifs for c-Ets. The transcription factor c-Ets is a member of the oncogene superfamily that binds to the $^{G}/_{C}{}^{A}/_{C}GGA^{A}/_{T}G^{T}/_{C}$ DNA motif (Woods *et al* 1992; Faisst and Meyer 1992) and regulates a variety of cellular promoter and enhancer elements (MacLeod *et al* 1992). This transcription factor has been demonstrated to form transcriptional complexes with Pit1 and activate the rat PRL promoter (Bradford *et al* 2000; Howard and Maurer 1995). In the turkey however, no studies have been done to investigate the possible binding of c-Ets and its possible role in the regulation of PRL gene transcription.

4.4 The PCR-SSCP analysis and characterisation of fragment B

A second primer pair B forward (CCTGGCCCCATTCTCATCCC) and B reverse (AGCAGTGTGCCCTTGCGGCC) amplified a 331bp DNA segment located from –1699 to –1370 in the tPRL promoter (Table 3.4). One of the three preliminary SSCP conditions (9% PAG at 8.5volt/cm for 7 h) tested to detect polymorphisms within the DNA fragment revealed three distinct single stranded DNA migration patterns. The result shows that one of the fragment B alleles has conformational changes indicated by its mobility shift during electrophoresis (Fig 4.4a). Accordingly, fragment B PCR amplified products from 62 birds were genotyped based on the migration pattern obtained using a 9% PAG at 8.5v/cm for 7h. Of the 62 birds

however, 7 samples were discarded because of inconsistency of their gel migration pattern during electrophoresis. Thus, the succeeding analyses of this fragment are primarily based on the genotype of 55 birds. The homozygous genotypes were designated as B_1B_1 and B_2B_2, and the heterozygotes were designated as B_1B_2 (Fig 4.4a). The genotypic and allelic frequencies within the sample population were calculated in Table 4.4a. The B_2B_2 homozygous genotype showed the lowest frequency at only 3%, and the B_2 allele is observed in 29% of the sample population.

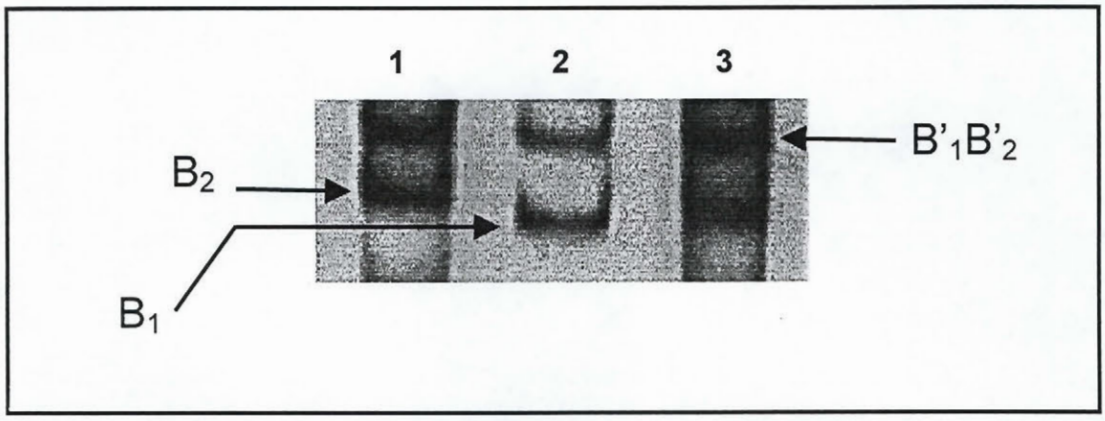


Figure 4.4a Detection of one-allele polymorphism in fragment B. The PCR-amplified fragment B samples were separated in 9% PAG at 8.5volt/cm for 7 h. The mobility shifts are between the two fragment B alleles designated as B_1 (lane 2) or B_2 (lane 1) while the conserved allele is designated as $B'_1B'_2$ (lane 3). Lanes 1 to 3 are genotypes B_2B_2, B_1B_1 and B_1B_2 respectively.

Genotypes	Frequency (broody)	Frequency (nonbroody)	Frequency population	Total no. in population	Haplotypes	Frequency (haplotypes)
B_1B_1	0.32	0.68	0.45	25	B_1	0.71
B_1B_2	0.18	0.82	0.51	23	B_2	0.29
B_2B_2	0.5	0.5	0.036	2		
				N=55		N=110

Table 4.4a Genotypic and allelic frequencies of fragment B polymorphism in the sample population

The plasma PRL concentration of the birds in this study was determined by the homologous radioimmunoassay (Guemene *et al* 1994). The three distinct genotypes determined using SSCP were designated as were B_1B_1 homozygotes (n=25), B_2B_2 homozygoyte (n=2) and B_1B_2 heterozygotes (n=28). Analysis of variance (ANOVA) was performed to identify the association between the genotypes (B_1B_1, B_1B_2 and B_2B_2) and the level of plasma PRL. Figure 4.4b shows the level of plasma PRL in the three genotypes. The ANOVA result indicated that there was an association between the B_1B_1 and B_2B_2 homozygote genotypes and levels of plasma PRL ($p \leq 0.05$) but not between the B_1B_1 and B_1B_2 genotypes ($p \geq 0.05$). Due to the low number of B_2B_2 birds compared to the B_1B_1 and B_1B_2 individuals, the effect of the genotypes on the level of plasma PRL was further investigated using the analysis of variance in the incubating and non-incubating birds subsets.

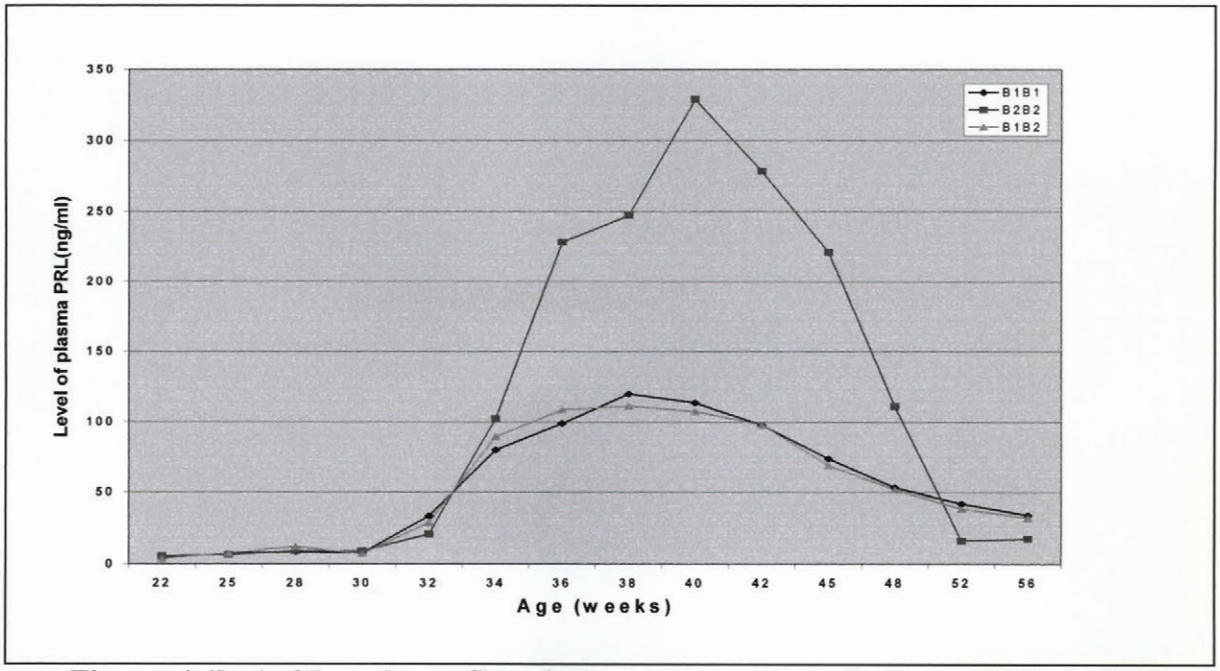


Figure 4.4b A 35-week profile of the concentration of plasma PRL in B_1B_1 (n=25), B_2B_2 (n=2) and B_1B_2 (n=28) individuals.

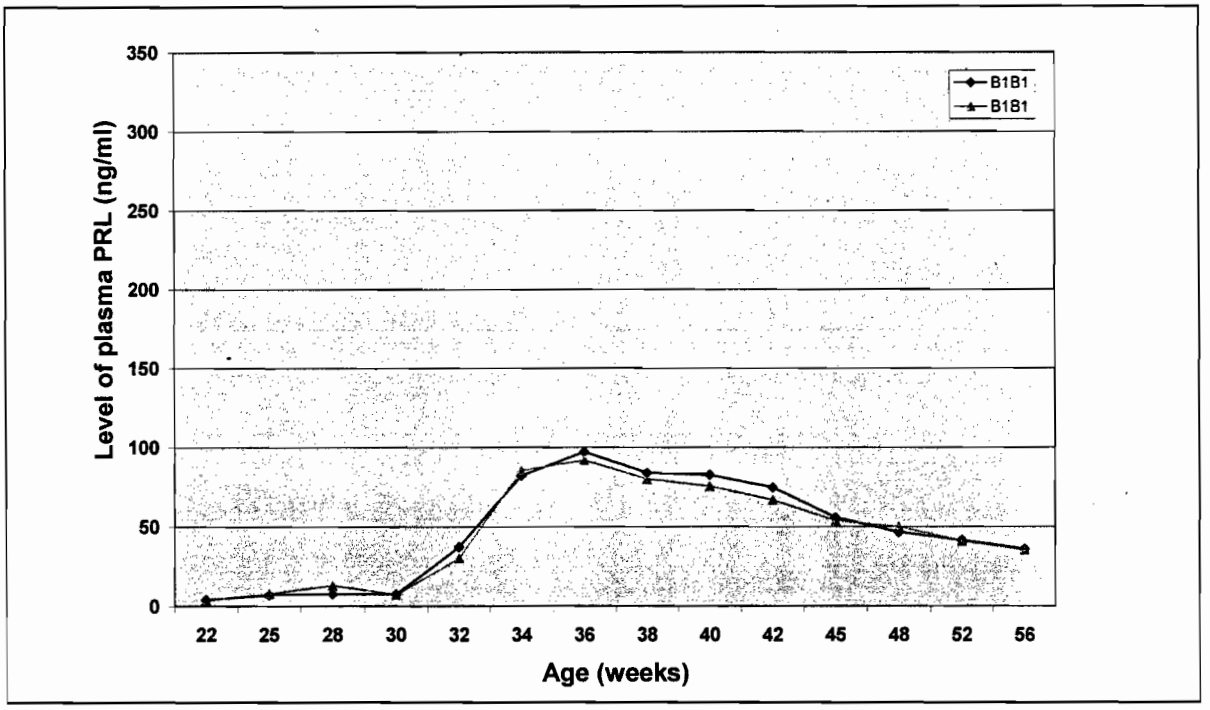


Figure 4.4c A 35-week profile of the concentration of plasma PRL in non-incubating B_1B_1 (n=17) and B_1B_2 (n=23) individuals.

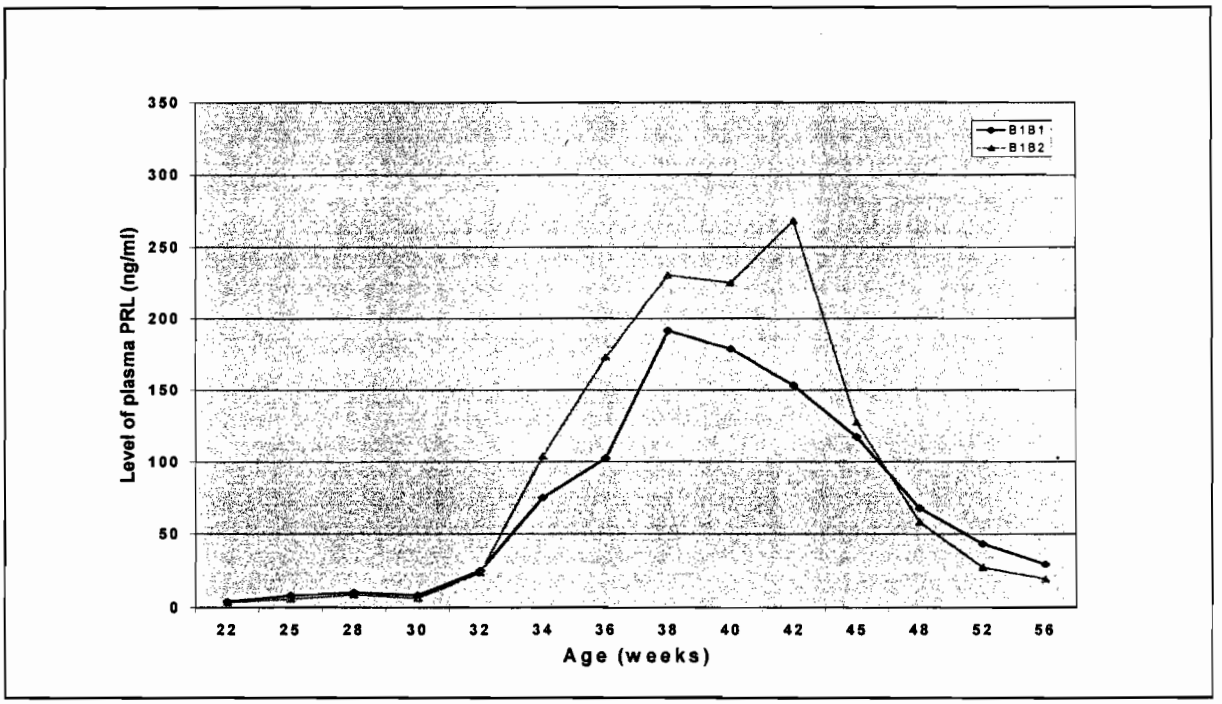


Figure 4.4d. A 35-week profile of the concentration of plasma PRL in incubating B_1B_1 (n=6) and B_1B_2 (N=7) individuals.

Subsequent analysis of the birds within the non-incubating subgroup revealed no association ($p \geq 0.05$) between the parameters tested within this subset. Figure 4.4c shows the plasma PRL profile of 40 non-incubating hens, 17 of which are B_1B_1 homozygotes and 23 are B_1B_2 heterozygotes. In the incubating subgroup however, the genotypes (B_1B_1, n=8 and B_1B_2, n=5) were associated ($p \leq 0.05$) with the levels of plasma PRL. The rapid increase in the concentration of plasma PRL in the B_1B_2 genotype within the incubating group during the 32nd week (Fig 4.4d) indicate that the genetic variation detected within fragment B may have influenced the concentration of plasma PRL during the 35-week period of the turkey reproductive cycle.

Sequence characterisation of fragment B from the same individuals selected for sequencing in the previous fragment (A) detected two base substitutions between the B_1B_1 and the B_2B_2 hens. The base changes were identified as C to T and T to C transitions at –1402 and –1400 upstream of the tPRL promoter respectively (Fig 4.4e). Comparison of the sequence results with reported tPRL promoter sequence (U05953) at –1699 to –1370 revealed that the B_1B_1 and B_2B_2 homozygous nucleotide sequences were 99.5% and 98.9% similar to the latter, indicating the possible strain-specific variations among the different turkey populations (Fig 4.4e). The change in one nucleotide base (at –1402) modified the sequence to one which sequence is similar to the complementary core sequence of human GATA1 transcription factor DNA-binding motif (WGATAR, Joulin *et al* 1991).

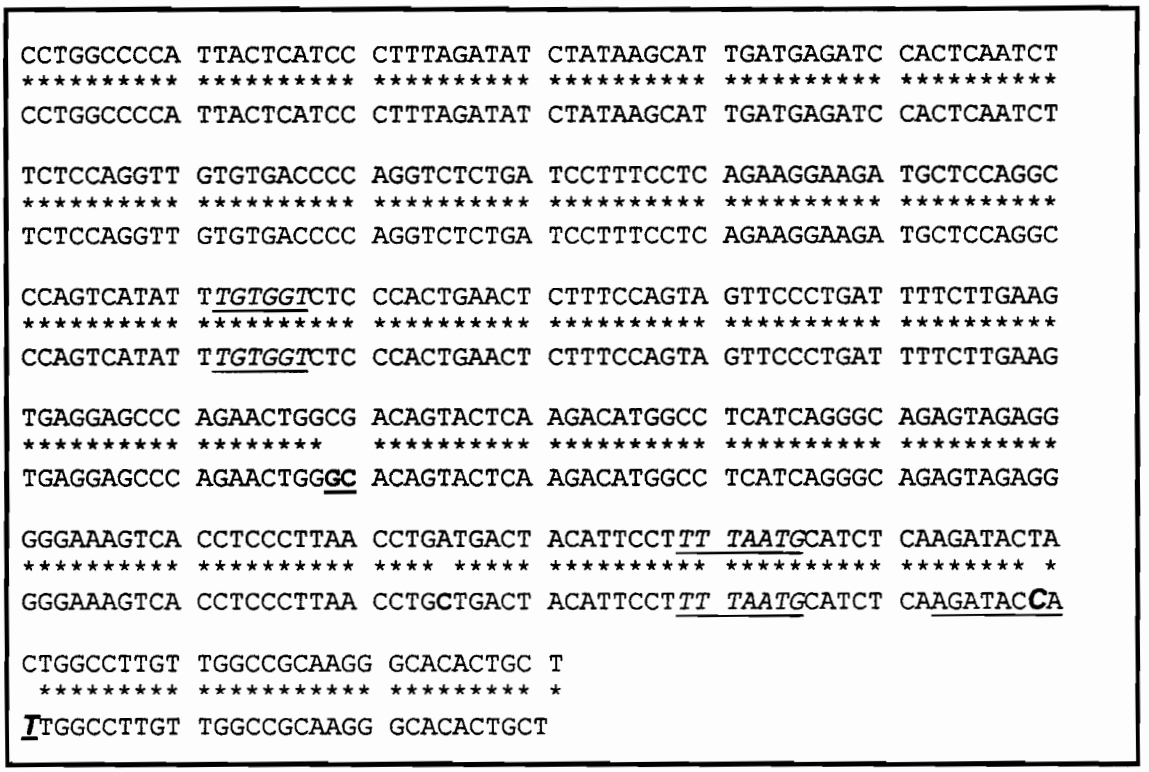


Figure 4.4e Sequence analysis of Fragment B. The upper sequence is the published tPRL gene promoter sequence (U05953) from –1699 to –1370; the lower sequence is the variations detected in this study and the nucleotide base changes are marked in **bold** italics Arial letters. The bases marked in **bold and underlined** are the strain-specific variation, while the bases marked in **bold italics** are the base substitutions detected in this study. The underlined sequence is similar to the DNA-binding motif for GATA1 transcription factor. The DNA binding motif with high similarity to CdxA binding sites are in *underlined italics*.

The transcription factor GATA1 is the first member of a zinc-finger family of DNA-binding proteins demonstrated to be the major sequence-specific DNA-binding protein of hematopoietic cell lineage in mammals (Joulin *et al* 1991). In recent studies, the GATA family of transcription factors was found to require transcriptional cofactors (*i.e.* Pit1) and may act as "docking sites" in important regulatory elements in the promoter region (Dasen *et al* 1999). Furthermore, Dasen *et al* 1999 demonstrated that GATA2 determine the position of the different pituitary cell types before their terminal differentiation.

In avian species, very few studies have been conducted to identify functional transcription factor binding sites. The detection of a potential GATA1 site in this study however, may indicate that this region could bind GATA1 which may then play a role in cell differentiation or may interact with other transcription factors (*i.e.* Pit1) and regulate tPRL gene transcription.

Further analysis of the nucleotide sequence using a transcription factor databank compiled by Heinemeyer *et al* 1998 showed that this region also contains short DNA motifs, which are 91% identical to CdxA binding sites. The transcription factor CdxA is a homeobox protein involved in the differentiation of embryonic cells of endodermal origin, which has been demonstrated to bind to the DNA consensus motif $A^{A}/_{T}T^{A}/_{T}AT^{A}/_{G}T$ (Margalit *et al* 1993). To date however, no studies have been done to link these transcription factors to PRL gene regulation or pituitary cell proliferation.

Overall, the sequence variation in fragment B appears to increase the concentration of plasma PRL in individuals with the B_2 haplotype possibly by the modification of a transcription factor-binding site. Higher levels of plasma PRL increase the frequency of broodiness in commercial turkey flocks. Therefore, this behaviour was selected against in the traditional breeding programs. The overall genotypic and allelic frequencies reflect the systematic selection against broodiness and possibly the B_2 haplotype.

4.5 The PCR-SSCP analysis and characterisation of Fragment C

The primer pair C_F and C_R amplified a 309bp DNA segment at −1369 to −1050 upstream of the transcription start site and was designated as Fragment C (Fig 3.4). The size of the expected fragment (320bp) did not match the size of the fragment obtained (309bp). This disparity in the fragment size may be attributed to deletions that occurred within fragment C. Two of the three preliminary SSCP conditions (Table 3.5) tried on this fragment revealed a change in the migration speed of the single-stranded DNA of some individuals. The migration pattern indicated a two-allele SSCP and these alleles were subsequently designated as the C_1 and C_2 alleles (Fig 4.5a).

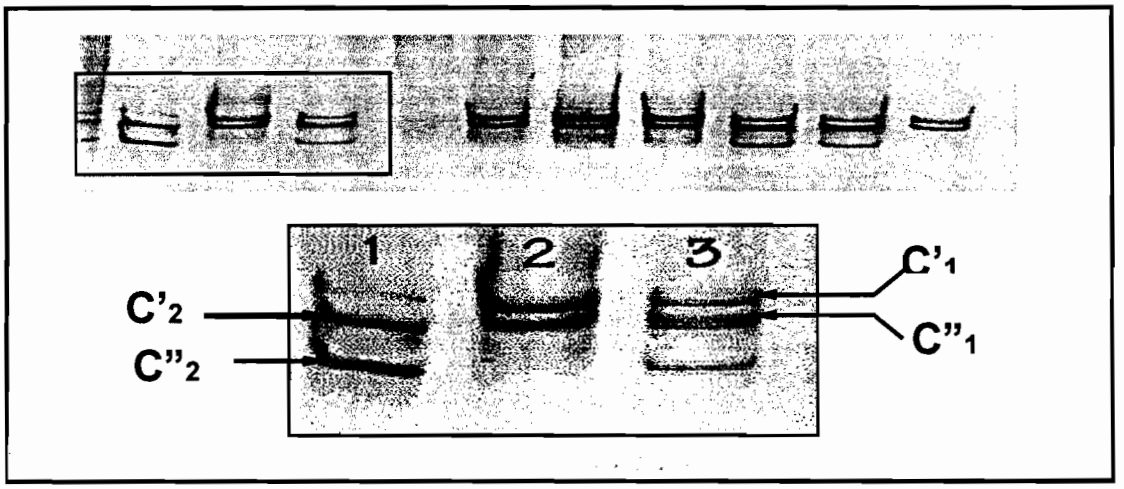


Figure 4.5a Detection of a two-allele SSCP in fragment C. The PCR amplified fragment C samples were separated in 9%PAG at 8.5v/cm for 7 h, room temperature. Inset lanes 1 to 3 are genotypes C_2C_2, C_1C_1 and C_1C_2 respectively.

A total of 62 individuals were genotyped the results were designated as C_1C_1 (n=37) and C_2C_2 (n=2) homozygotes, and C_1C_2 (n=18) heterozygous individuals. Unlike the polymorphism detected in fragment B, both alleles in this fragment appear

to have different conformation as shown in Figure 4.5a. The C fragment genotypic and allelic frequencies are listed in Table 4.5a.

Genotypes	Frequency (broody)	Frequency (nonbroody)	Frequency (population)	Total no. in population	Haplotypes	Frequency (haplotypes)
C_1C_1	0.32	0.68	0.60	37	C_1	0.78
C_1C_2	0.22	0.78	0.37	23	C_2	0.22
C_2C_2	0.5	0.5	0.032	2		
				N=62		N=124

Table 4.5a Genotypic and allelic frequencies of Fragment C polymorphism in the sample population.

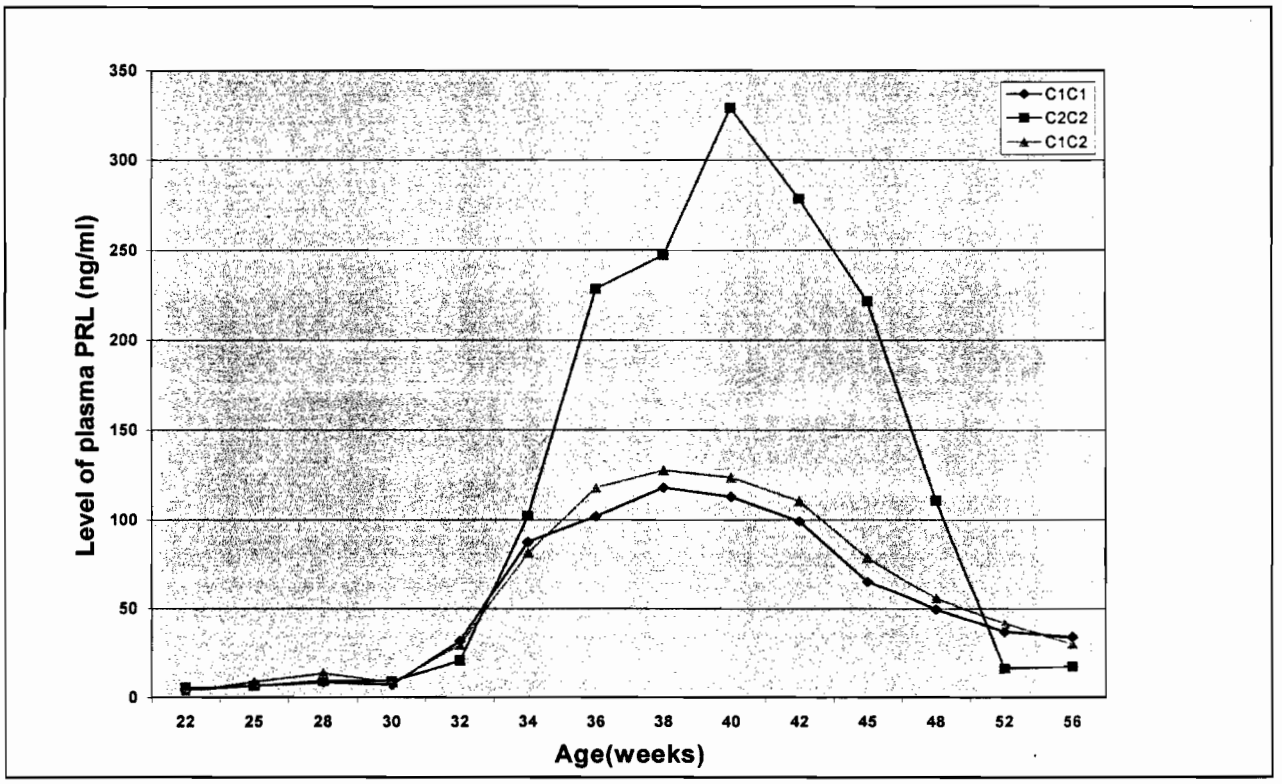


Figure 4.5b A 35-week profile of the concentration of plasma PRL in C_1C_1 (n=37), C_2C_2 (n=2) and C_1C_2 (n=23) individuals.

The effect of the C_1C_1 and C_2C_2 genotypes on the concentration of plasma PRL in 62 birds were assessed and an association ($p \leq 0.05$) between the parameters

was observed. The C_2C_2 homozygous genotype appears to be associated with higher concentration of circulating PRL compared to the C_1C_1 homozygous genotype. Figure 4.5b shows the level of plasma PRL of each of the three genotypes. The two individuals exhibiting the C_2C_2 were the same individuals with the B_2B_2 genotype, one of which demonstrated incubation behaviour while the other was not incubating. Due to the small number of C_2C_2 individuals, the association between this homozygous genotype and higher levels of plasma PRL observed in this study is not conclusive. To further evaluate the effect of the C polymorphism on the concentration of circulating PRL, the 62 birds were divided into the incubating and non-incubating subgroups.

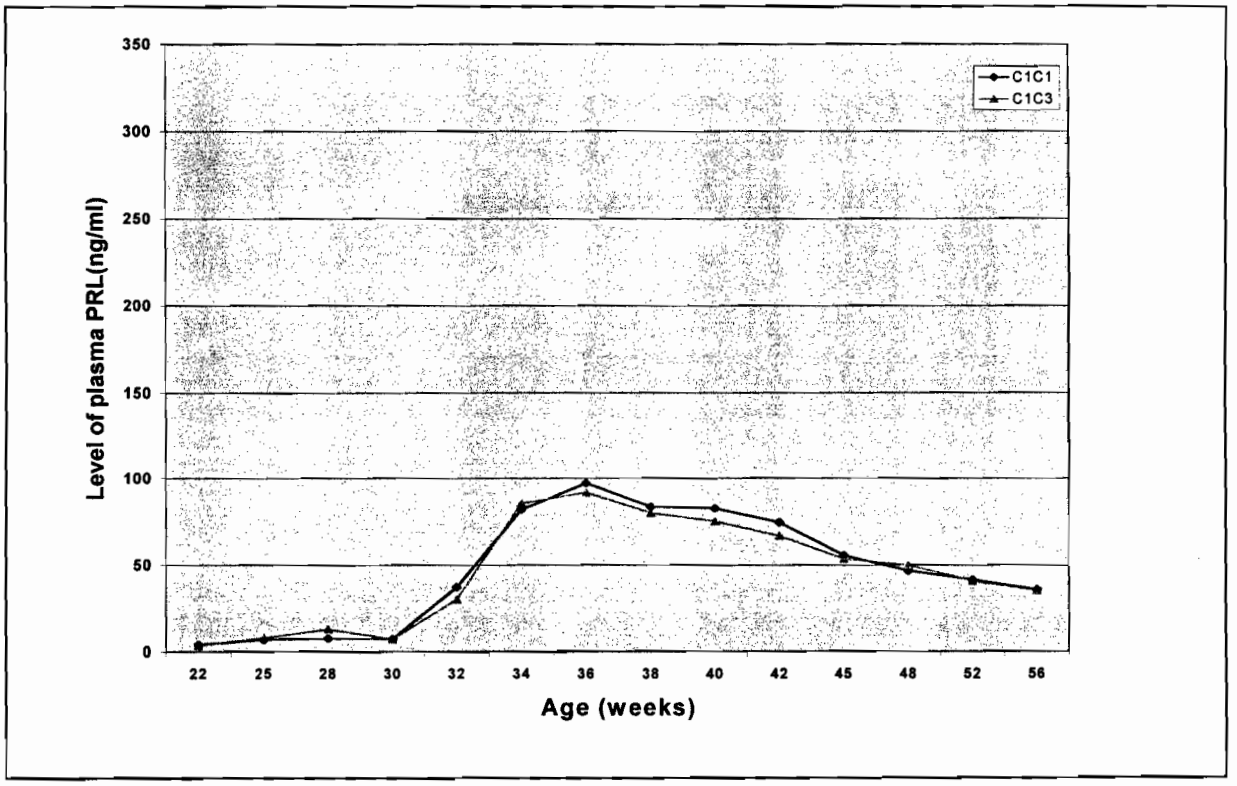


Figure 4.5c A 35-week profile of the concentration of plasma PRL in homozygous C_1C_1 (n= 25) and heterozygous C_1C_2 (n=18) nonbroody hens.

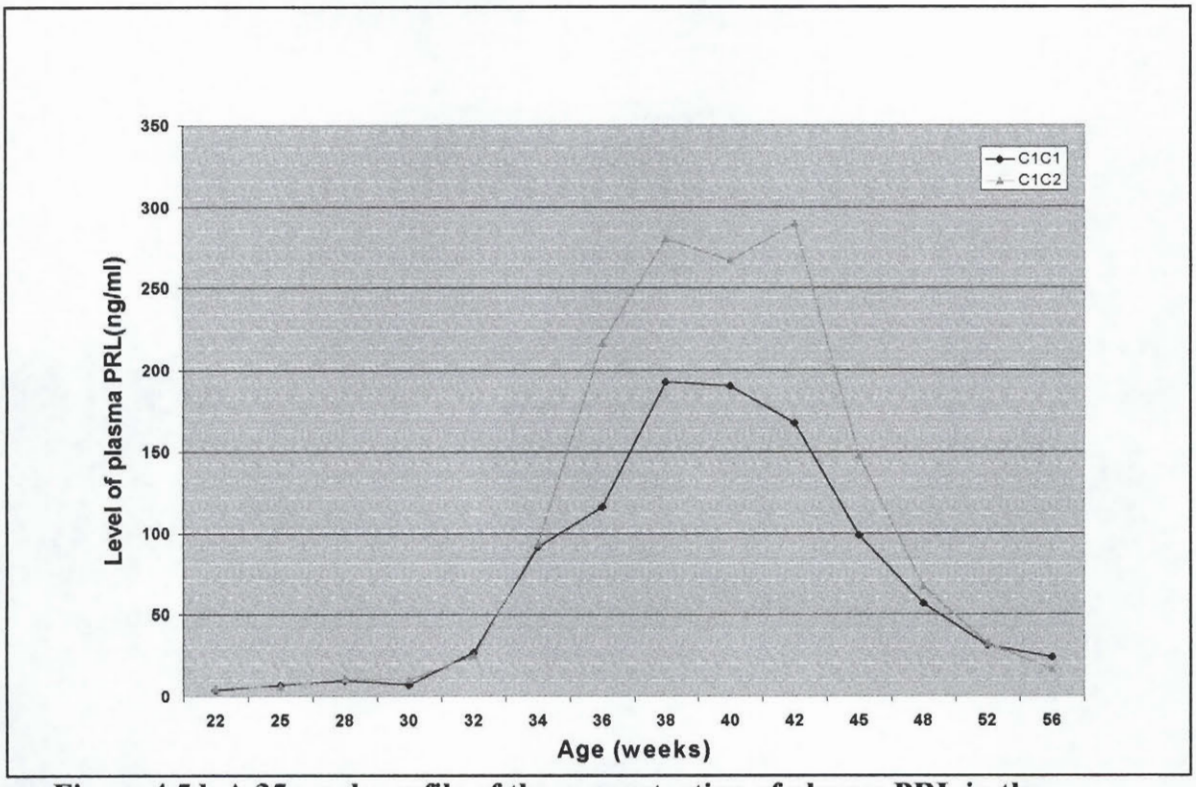


Figure 4.5d. A 35-week profile of the concentration of plasma PRL in the homozygous C_1C_1 (n=12) and heterozygous C_1C_2 (n= 5) broody hens.

Of the 62 birds investigated, 18 were observed to be incubating in nest boxes. Accordingly, the average level of plasma PRL during the 35-week turkey reproductive cycle in the incubating subgroup (Fig 4.5d) was higher that those of the non-incubating subgroup (Fig 4.5c). Therefore the PRL response to the C genotype was reassessed within these subsets. The genotypic effect on the levels of plasma PRL within the non-incubating subgroup (C_1C_1, n=25 and C_1C_2, n=18) was analysed and it showed no association ($p \geq 0.05$) between the parameters examined. In the incubating subgroup (C_1C_1, n=12 and C_1C_2, n=5) however, the analysis showed an association ($p \leq 0.05$) between the C_1C_1 (n=12) and C_1C_2 (n=5) genotypes and the levels of plasma PRL. Since only one C_2C_2 homozygous genotype was observed in

the sample population therefore it was not included in the statistical analysis. Table 4.5b shows an analysis of variance to measure the effect of fragment C genotypes on the 35-week plasma PRL concentration. The results indicate that the conformation polymorphism detected in SSCP analysis of the C fragment is associated with high plasma PRL concentration which may increase the likelihood of broodiness in turkey hens. However, too few individuals were observed in this class (n=5) to make a definitive conclusion.

Groups	Df	SS	MS	F	P
Genotypes	1	40447.704	40447.704	15.884	<0.0001
Weeks	15	824799.452	63446.112	24.915	<0.0001
Gen X Wk	15	78989.091	6076.084	2.386	<.0062
Error	148	376883.790	2546.512		

Table 4.5b. Analysis of variance between fragment C genotypes and the concentration of plasma PRL during a 35-week period (n=15).

To further elucidate the association between C genotypes and broodiness, the polymorphism within the fragment was characterised. Sequence analysis of fragment C showed four mutations in the C_2C_2 genotype, a 12bp deletion at –1282 to –1271; a C insertion at –1246, A to G base substitution at –1206 and a T to C transition at –1184. The 12bp (AACGCAAGCAGT) deletion appears to contain a putative avian Pit1 binding motif ($^{T}/_{A}$NCTNCAT; Ohkubo *et al* 1996) on the antisense strand (ATGNAGN$^{T}/_{A}$). Furthermore, the C insertion at –1246 seems to alter another potential Pit1 binding site on the sense strand. The C_1C_1 genotype has a

T_CCTTCATA at –1247 to –1238 while the C_2C_2 genotype has a TCCCTTCATA at the same location (Fig 4.4e). The G to A transition on the other hand contains a *Msp1* (CC↓GG) restriction site. The 12bp deletion and the C insertion explains the 11bp disparity between the expected and the PCR amplified fragment obtained in this study.

```
AGCCCACGGT CAACCTGTTG TCTACCAGGA CATCTAGGTC TTCTCAGCAG AGCTCCTTTC
********** ********** ********** ********** ********** **********
AGCCCACGGT CAACCTGTTG TCTACCAGGA CATCTAGGTC TTCTCAGCAG AGCTCCTTTC

CAGCAGGTCA GCCCCCAGCC TGTACTAACG CAAGCAGTAA AAAAGGTCTA CCTTTTTTCT
********** ********** *****              ** ********** **********
CAGCAGGTCA GCCCCCAGCC TGTACT---- --------AA AAAAGGTCTA CCTTTTTTCT

CC-TTCATAG AATCACAGAA TTGTAGGGGT AGGAGGGAAC CTCCGGAGAT CATCTAGTCC
** ******* ********** ********** ********** **** ***** **********
CCCTTCATAG AATCACAGAA TTGTAGGGGT AGGAGGGAAC CTCCAGAGAT CATCTAGTCC

AACCACTCTG CCAAATCATC ACAGTAACAC AGGAAAGTGT CTGGTGGGTT TTGTTTATTA
****** *** ********** ********** ********** ********** **********
AACCACCCTG CCAAATCATC ACAGTAACAC AGGAAAGTGT CTGGTGGGTT TTGTTTATTA

TTGCTTCAAA AAGCACAGCC ACAGTTACGA AATAATGGGA GATTCAGGAT TATACACATA
********** ********** ********** ********** ********** **********
TTGCTTCAAA AAGCACAGCC ACAGTTACGA AATAATGGGA GATTCAGGAT TATACACATA

CCTGTTCCAC ATGTACAGAA C
********** ********** *
CCTGTTCCAC ATGTACAGAA C
```

Figure 4.5e Sequence characterisation of C fragment polymorphism. A comparison between line 1, the reported tPRL gene promoter nucleotide sequence (U05953) at –1699 to –1323 and line 2, C_2C_2 homozygous individual at the same position. The polymorphisms detected are in **bold** while the putative Pit1 binding DNA motifs are in **bold and underlined** and the potential CdxA binding consensus sequences are in ***bold italics.***

Further analysis of the sequence revealed that the three putative Pit1 binding sites reported in chPRL by Ohkubo *et al* (personal communication) are within this

region (–1298 to –1081). The most distal of the three is located at the antisense strand from –1279 to –1272, the second is at the sense strand from –1250 to –1243 and the third is from –1096 to –1088 of the antisense strand. The characterisation of fragment C polymorphisms subsequently revealed that two of the three putative Pit1 binding sites were either deleted or altered. Taken together with the genotypic effect on levels of plasma PRL, the loss or modification of Pit1 binding sites in this region may significantly increase the plasma tPRL concentration in broody hens. However, any increase in the circulating levels of PRL appears to be in contrast with the role of Pit1 in mammalian PRL gene expression regulation.

In mammals, the regulation of transcription of the PRL gene has been attributed to the action of Pit1 binding to *cis*-acting elements upstream of its promoter (Ingraham *et al* 1988). The Pit1-binding motif referred to in these mammalian studies were located distal (–1713 to –1598; Day and Maurer 1989) and proximal (–190 to –120, Iverson *et al* 1990) to the transcription start site. Mutation(s) at Pit1-binding motif of the mammalian PRL gene proximal to transcription start site, particularly P1, P2, and P3 (Nelson *et al* 1986) appears to disrupt the PRL transcription complex (Iverson *et al* 1988). Furthermore, Day and Maurer (1989) demonstrated that mutations in the distal Pit1-binding site particularly 3D, also reduce basal PRL activity.

In avian species, Pit1 binding sites in PRL promoter were located at 1280 to 1081 upstream of the chPRL gene. This region was demonstrated to bind chicken pituitary nuclear extracts as well as cPit1 expression vector transfected COS-7 cells as shown by gel mobility shift analysis (Ohkubo *et al* personal communication).

Sequence analysis of this DNA segment contains short DNA motifs identical to Pit1-binding consensus sequence reported for teleost and birds (Ohkubo *et al* 1996). Ohkubo *et al* (personal communication) demonstrated that the gene activity was high when the –1366 to –1255 upstream region was deleted indicating a possible inhibitory function.

Interestingly, the putative Pit1-binding motifs detected in turkey are similar to that observed in the chicken. In the C_2C_2 genotype, two of three potential Pit1 binding sites were mutated. One located at –1279 to –1272 was completely deleted while the other at –1250 to –1243 appears to be altered due to a C insertion at –1249. Although no experiment on the tPRL promoter activity was performed, results in this study indicate that at least two potential Pit1-binding sites within fragment C in turkey could suppress PRL gene expression. When present, these putative Pit1 binding sites could compete with other Pit1 enhancer elements for Pit1 transcription factor thereby reducing PRL gene transcription. This assumption is supported by the significant effect of the C_2 haplotype on plasma PRL concentration in broody hens (Figure 4.5d and Table 4.5b). In this study, only a few number of homozygous C_2C_2 birds (n=2) were available to statistically assess the effects of the homozygous genotype. In addition to Pit1, short DNA sequences with 92.1 to 98.6% similarity to Cdx A were observed in the C fragment.

4.6. The PCR-SSCP analysis and characterisation of fragment D

The PCR amplification using D_F (ACAGTTACGAAATAATGGGAG) and D_R (GTCGTAATCAGTGGGAATCTG) primer pair amplified a 301bp DNA

segment at –1110 to –810 upstream of the tPRL transcription start site. The PCR amplified DNA fragment was designated as Fragment D in Fig 3.4. A total of six electrophoretic conditions were used for the SSCP analysis (Table 3.5) but none of the conditions detected a polymorphism. These results may indicate that either the conditions tested were not sensitive enough to detect conformational changes or no sequence variation within fragment D exists among the individuals investigated.

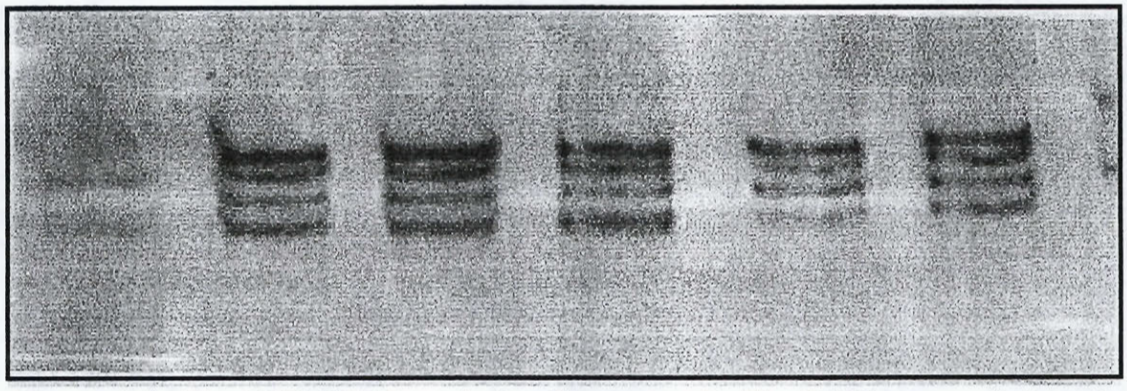


Figure 4.6a The PCR-SSCP analysis of fragment D. The SSCP was carried out in 12% PAG at 8.5v/cm for 10h. No polymorphism in fragment D was detected.

As in the previous fragments (A, B, and C), two PCR amplified fragment D samples were sequenced using the dideoxy chain termination protocol. The result obtained from both individuals revealed identical nucleotide sequence to the reported tPRL promoter nucleotide sequence (U05953) at the –1110 to –810 upstream of the transcription start site (Figure 4.6b). The direct sequence result appears to support the preceeding SSCP outcome, showing that no polymorphism can be detected at this particular tPRL gene DNA segment. The fragment D sequence result was further investigated through transcription factor search of the current transcription factor

databank (Heinemeyer *et al* 1998). The search revealed that the D fragment contains short DNA sequences with 90.4 to 98.6% similarity to several transcription factor binding consensus sequences. The binding motifs observed within fragment D include the previously described CdxA, as well as Oct1 (ATGCAAAT, Scholer *et al* 1989), and C/EBP (ATTGCGCAAT) or CCAAT/enhancer binding protein (Johnson 1993).

ACAGTTACGA **AATAATG**GGA GATTCAGGAT TATACACATA CCTGTTCCAC ATGTACAGAA
********** ********** ********** ********** ********** **********
ACAGTTACGA **AATAATG**GGA GATTCAGGAT TATACACATA CCTGTTCCAC ATGTACAGAA

CAAGTTGTCT AGAGGCAAGA AAATTCATTA ACACTGTATA CCT***TATTCAT*** ***TATGAT***CATC
********** ********** ********** ********** ********** **********
CAAGTTGTCT AGAGGCAAGA AAATTCATTA ACACTGTATA CCT***TATTCAT*** ***TATGAT***CATC

TAATTTAGAA GGTCTTTTCG TG**GATAAAT**G CATCTGAGAA ACAGATGAGA GATTACGCAT
********** ********** ********** ********** ********** **********
TAATTTAGAA GGTCTTTTCG TG**GATAAAT**G CATCTGAGAA ACAGATGAGA GATTACGCAT

TTGCTAACAT ATTCGTGCAG ATGAACCTCA CACAACAAGA AAACAGGGCC AACCTGCTGA
********** ********** ********** ********** ********** **********
TTGCTAACAT ATTCGTGCAG ATGAACCTCA CACAACAAGA AAACAGGGCC AACCTGCTGA

AGCTAGGTTG CAGATTACCA CAGACACATT AGATCAGGAA TCAGATTC***CA*** ***CTGATTACGAC***
********** ********** ********** ********** ********** **********
AGCTAGGTTG CAGATTACCA CAGACACATT AGATCAGGAA TCAGATTC***CA*** ***CTGATTACGAC***

Figure 4.6b Sequence analysis of fragment D. The first line represents the reported tPRL promoter sequence at –1110 to –810 (U05953) and the second line is the nucleotide sequence at the same segment of the tPRL promoter in this study. The short DNA motifs in **bold**, ***bold italics*** and underlined show high homologies to CdxA (Margalit *et al* 1993), Oct1 (Scholer *et al* 1989) and C/EBP (Johnson 1993) binding consensus sequences respectively.

The transcription factor Oct1 acts as a Pit1 coactivator and their interaction leads to anterior pituitary cell differentiation (Voss *et al* 1991). Likewise, the subsequent formation of a Pit1/Oct1 transcription complex was also shown to

regulate the expression of pituitary hormone gene expression, including PRL in rat (Diamond *et al* 1999). The transcription factor C/EBP was also demonstrated to regulate PRL gene expression (Jacob and Stanley 1999). Despite the lack of information on the potential transcription factor binding sites in the tPRL promoter, the presence of the DNA binding motifs could provide insights on the regulation of tPRL gene transcription.

4.7 The PCR-SSCP analysis and characterisation of fragment E.

The primer set E_F and E_R amplified a 354bp DNA segment located at –839 to –485 of the tPRL promoter region designated as fragment E (Table 3.4). A total of six preliminary SSCP conditions were performed on the PCR products but no clear resolution was achieved using any of the conditions (Table 3.5). In SSCP, mobility shifts are significantly influenced by the nucleotide sequence in the DNA strand particularly the neighbouring sequence over and above the base change (Glavac and Dean 1993). Modifications in the flanking sequence could alter the SSCP sensitivity in detecting the mutation (Sheffield *et a l* 1993). A second base change in or around the vicinity of one mutation could subsequently increase the difficulty of interpreting the mobility shifts observed.

Accordingly, before any other SSCP conditions were examined, two individuals were selected for sequence analysis. These individuals were selected based on a possible linkage of polymorphisms that were observed in fragments B and C. As anticipated, the sequence results indicated two base changes within 74bp of each other (Figure 4.7a). One polymorphism was an A to G transition at –728 and a T deletion at –654 upstream of the transcription site. The former was subsequently

determined to be within a *Hind III* restriction site (A↓AGCTT). Therefore, restriction fragment length polymorphism (RFLP), a tool in detecting base changes within restriction enzyme cutting sites, was carried out to genotype the sample population. To be consistent with the SSCP nomenclature in fragments B and C, the homozygous +/+ genotype was designated as E_1E_1; the -/- as E_2E_2, and the heterozygous genotype +/- as E_1E_2 (Fig 4.7b).

```
GATCAGGAAT CAGATTCCAC TGATTACGAC AGCATATACT GTGATTATGG TGGACATGCA
********** ********** ********** ********************** **********
GATCAGGAAT CAGATTCCAC TGATTACGAC AGCATATACT GTGATTATGG TGGACATGCA

CATCTTTTAC GCAAAGAATT TTCATATATA GAAAATGATT TCATGGTTCG GAAGCTTTTA
********** ********** ********** ********** ********** * ********
CATCTTTTAC GCAAAGAATT TTCATATATA GAAAATGATT TCATGGTTCG GGAGCTTTTA

AAATAATGCT GATTTAATTA CAAAATGTTT ATGATTAAAC AGTAAGCATA CAAATTCTTC
********** ********** ********** ********************** **********
AAATAATGCT GATTTAATTA CAAAATGTTT ATGATTAAAC AGTAAGCATA CAAATTCTTC

CTCTTTGTTG TTACAAATTA TTACTTTTTT AATGACAACT GTCCCTGTTT CTCAACTTAT
***** **** ********** ********** ********************** **********
CTCTTCGTTG TTACAAATTA TTACTTTTTT AATGACAACT GTCCCTGTTT CTCAACTTAT

CTCATCCTTA GTACCAGTTA TATCATTATC TGTTGGTAAA TAATATCCTT TTTAGCTGTA
********** ********** ********** ********************** ** *******
CTCATCCTTA GTACCAGTTA TATCATTATC TGTTGGTAAA TAATATCCTT TT-AGCTGTA

TGGAGACAAA CACACACTAC GTATAATAAT GACCTGTCTT TCCAGAAGCC TCCAT
********** ********** ********** ********************** *****
TGGAGACAAA CACACACTAC GTATAATAAT GACCTGTCTT TCCAGAAGCC TCCAT
```

Figure 4.7a Sequence analysis of fragment E. First line is the reported tPRL gene promoter sequence at –839 to –485 (U05953), also the +/+ or the E_2E_2 homozygote, and line two is the nucleotide sequence at the same region obtained in this study (also the -/- or the E_1E_1 homozygous genotype). Base changes are in **bold** or _. The DNA motifs showing high homologies to CdxA, Oct1, and GATA1 are marked in underlined ***bold Italics*** and enclosed in a box respectively.

Analysis of fragment E sequence revealed the segment contains several short DNA motifs with up to 98.6% similarity to several known TF binding sites. The

consensus sequence homologies to CdxA, Oct1 and GATA1 binding sites are 91.4 to 98.6%, 90.9 to 92.7% and 91.8% respectively.

To test linkage between the two point mutations detected by sequence analysis, *Hind III* digested PCR products were tested in three additional SSCP conditions. The mobility pattern was the same for all E_1E_2 heterozygotes as with the banding pattern for E_1E_1 and E_2E_2 homozygous individuals (Fig 4.7c.). The result indicates that the base changes within fragment E is linked. The inconsistency and the low repeatability of the SSCP result from undigested PCR products shown in Figure 4.7b could be due to the position of both base changes and the base composition flanking the polymorphic sites.

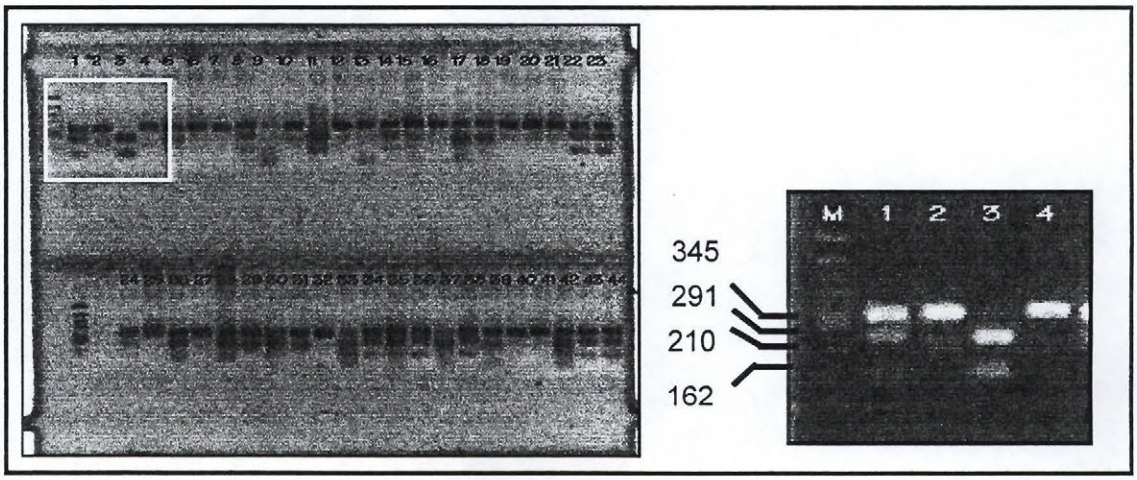


Figure 4.7b PCR-RFLP analysis of fragment E using *Hind III* restriction enzyme. The *Hind III* fragment E digests were separated in 1% agarose gel and electrophoresed at 100v for ~20min. In the inset, Lane M was loaded with ϕx174 *Hinc II* digest 100bp molecular marker while lane 1 is the +/- (or E_1E_2) heterozygote, lane 3 is the +/+ (or E_1E_1) homozygote and lanes 2 and 4 are the -/- (or E_2E_2) homozygous individuals.

Unlike the polymorphisms detected in fragments B and C, it is the E_2E_2 genotype that is entirely identical to the reported sequence (U05953). The A to G base change in the E_1E_1 genotype is identical to the nucleotide base of the chPRL gene at the same location (Ohkubo *et al* personal communication) while the C to T does not match either of the chPRL or the reported tPRL gene promoter sequences (Fig.4.7c).

The SSCP results of the *HindIII* digested fragment E PCR products showed that the two polymorphic sites are linked within this fragment. The genotypic and allelic frequencies listed in Table 4.7 were based on the genotypes E_1E_1 (-/-); E_2E_2 (+/+), and E_1E_2 (+/-).

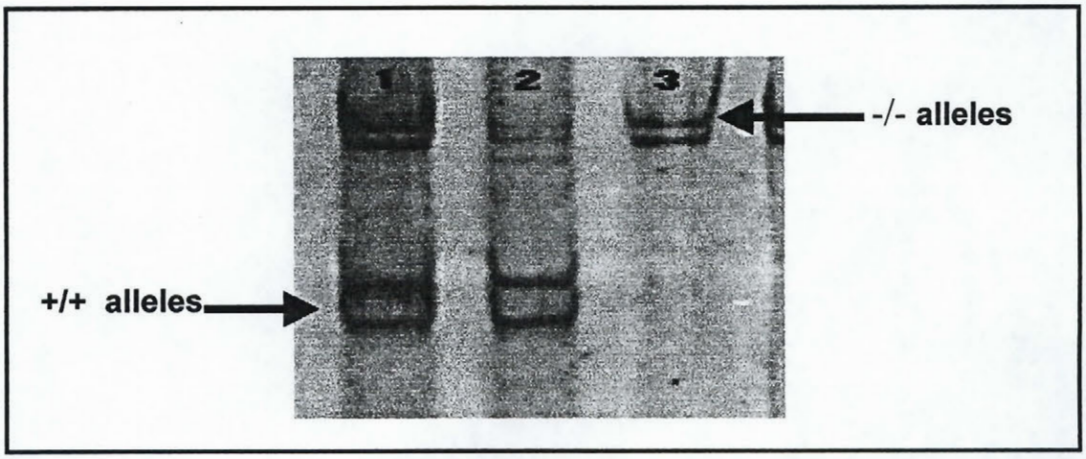


Figure 4.7c The PCR-RFLP-SSCP analysis of fragment E *Hind III* digests. The *Hind III* digested samples were separated in 8% PAG at 8.5v/cm for 6 h. Lane 1 contain +/- heterozygote (E_1E_2), lane 2 the +/+ homozygote (E_1E_1) and lane 3 with the -/- homozygote (E_2E_2). No additional polymorphism was detected from the banding pattern observed. These samples were the same individuals in lanes 1,3,and 2 in the inset picture of Fig 4.7b.

The three genotypes obtained using RFLP were E_1E_1 homozygote (n=35), E_2E_2 homozygote (n=2) and E_1E_2 heterozygote (n=25). Analysis of variance show

that the E_2E_2 homozygous genotype is associated with higher levels of plasma PRL than the homozygous E_1E_1 genotype. Upon further analysis however, no significant difference was observed in the concentration of circulating PRL between the homozygous E_1E_1 and E_1E_2 heterozygous individuals ($p \geq 0.05$) during the 35-week period of the turkey reproductive cycle. To further evaluate the effects of the E genotypes on the levels of plasma PRL, the genotypes were grouped into incubating (n=17) and non-incubating (n=43) hens. Out of the 17 incubating hens, 10 were of the the E_1E_1 homozygous genotype and 7 were of the E_1E_2 homozygous genotype. In the non-incubating group, there were 25 E_1E_1 and 18 E_1E_2 genotypes. Only two individuals (1 incubating and 1 non-incubating) in the population were E_2E_2 homozygous genotype and were not included in the statistical analysis. The analysis of variance performed with the incubating and non-incubating subgroups showed that there was no association ($p \geq 0.05$) between the genotype and the levels of plasma PRL within either subroup. The complete linkage of the E_2E_2 genotype with the B_2B_2 and C_2C_2 genotypes in the two individuals may explain its association with a higher level of plasma PRL.

Genotypes	Frequency (broody)	Frequency (nonbroody)	Frequency (population)	Total no. in population	Haplotypes	Frequency (haplotypes)
E_1E_1	0.29	0.71	0.57	35	E_1	0.77
E_1E_2	0.28	0.72	0.40	25	E_2	0.23
E_2E_2	0.5	0.5	0.03	2		
				N=62		N=124

Table 4.7 The genotypic and allelic frequencies of fragment E polymorphism in the sample population.

4.8 The PCR-SSCP analysis and characterisation of fragment F

The sixth tPRL promoter section analysed was a 319bp DNA region located at –538 to –240 upstream of the transcription start site (Fig 3.4). The fragment was designated as fragment F and was subsequently amplified using the oligonucleotide sequence GGAGACAAACACACACTACG as the forward primer (F_F) and the sequence GAGTATGGCTGGATGAAGAG as the reverse primer (F_R) (Table 3.4). After experimenting with five SSCP conditions (Table 3.5), one condition (8% PAG + 5% glycerol at 8.5v/cm for 10h) revealed a clear resolution for detecting polymorphism within fragment F and this condition was subsequently used to genotype the 66 birds. The mobility shift observed was attributed to the conformational modification of one of the alleles (Fig 4.8a). To be consistent with the nomenclature used in the previous fragments, the homozygous individuals were designated as F_1F_1 and F_2F_2 while the heterozygotes were designated as F_1F_2.

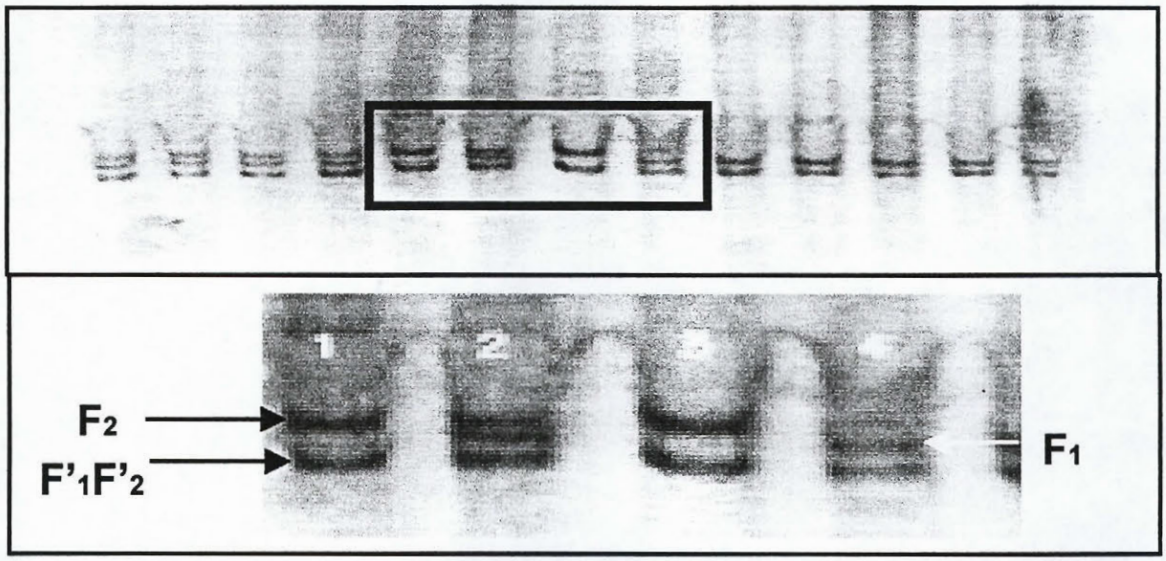


Figure 4.7a Detection of one-allele polymorphism in fragment F. The PCR amplified fragment F were separated in 8% PAG + 5% glycerol at 8.5v/cm for 10h at room temperature. Inset lanes 1 and 3 are homozygous F_2F_2 individuals, lane 2 is heterozygous F_1F_2 and lane 4 is F_1F_1.

The genotypic and allelic frequencies for F fragment are listed in Table 4.8. The sample population is made up of 50% homozygous F_1F_1 and 47% heterozygous F_1F_2 hens. Assessment of incubation behaviour among individuals with different genotypes show that in birds with the F_1F_2 heterozygous genotype 31% appears to be broody. In the F_1F_1 homozygous genotype 26% were broody and 50% in the F_2F_2 homozygous genotype express this maternal behaviour. In chicken, the polymorphisms in this location appear to differentiate an incubating from non-incubating strain (Kansaku, personal communication).

Genotypes	Frequency (broody)	Frequency (nonbroody)	Frequency (population)	Total no. in population	Haplotypes	Frequency (haplotypes)
F_1F_1	0.26	0.74	0.50	37	F_1	0.734
F_1F_2	0.31	0.69	0.47	27	F_2	0.266
F_2F_2	0.5	0.5	0.03	2		
				N=62		N=124

Table 4.8 The genotypic and allelic frequencies of fragment F polymorphism in the sample population.

Of the 62 birds available for the statistical analysis, 31 were F_1F_1 homozygous, 2 were F_2F_2 homozygous and 29 were F_1F_2 heterozygous individuals. The analysis of variance performed to test the genotypic effect on levels of plasma PRL revealed significance between the two homozygous genotypes ($p \leq 0.05$). There were only two F_2F_2 individuals and they were the same hens showing the B_2B_2, C_2C_2, and E_2E_2 homozygous genotype. Thus F_2F_2 individuals were not included in the subsequent statistical analysis. The analysis of variance was expanded to test the

association of genotypes within subgroups based on the physiological state of the hens. The individuals were divided into the incubating (n=17) and non-incubating (n=43) hens. There were 8 F_1F_1 and 9 F_1F_2 in the incubating hens, while 23 F_1F_1 and 20 F_1F_2 were placed in the non-incubating subgroup. The results showed that there was no association ($p \geq 0.05$) between the parameters tested within each of the subgroups.

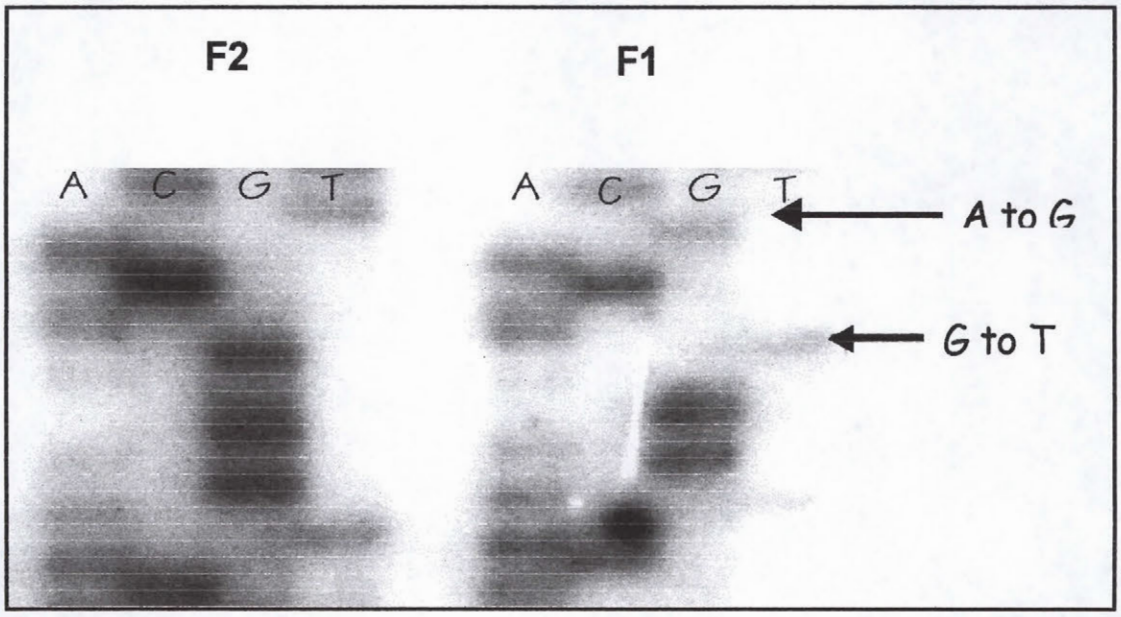


Figure 4.8b. Characterisation of fragment F polymorphism. An A to G transversion and a T to C transition at –319 and –323 respectively were detected in the tPRL promoter region.

Fragment F was sequenced by the cycle sequencing using $\delta^{32}P$ labelled primers. The result shows that two base substitutions have occurred at –323 and –319 of the tPRL promoter in the F_2F_2 genotype nucleotide sequence. The first was a G to T transversion and the second was an A to G transition at –323 and –319 respectively (Fig 4.8b). Accordingly the sequences obtained were compared with the reported tPRL sequence at –528 to –240 upstream of the tPRL promoter (U05953). Like in

fragment E, the F_2F_2 genotype appears to be identical to that of the reported tPRL promoter sequence at this particular region (Fig.4.8c).

```
GGAGACAAAC ACACACTACG TATAATAAT GACCTGTCTTT CCAGAAGCCT CCATTCACAT
********** ********** ********** ********************** **********
GGAGACAAAC ACACACTACG TATAATAAT GACCTGTCTTT CCAGAAGCCT CCATTCACAT

TCTCTGGATC AACTTCAGTA CAATTCCTAT TCTTTCTCTT ACTGTAGAAA TTGTATTATT
********** ********** ********** ********************** **********
TCTCTGGATC AACTTCAGTA CAATTCCTAT TCTTTCTCTT ACTGTAGAAA TTGTATTATT

TCCTTTCCAG AAATAGCTAG AATTGGAGGG TGAAGAGACA AGGAAGAAAC AGAAGATATC
********** ********** ********** ********************** **********
TCCTTTCCAG AAATAGCTAG AATTGGAGGG TGAAGAGACA AGGAAGAAAC AGAAGATATC

TGCAGGGATG AACAACATTT TATAAACATA GAGGAGAACA ATCTCAGAAC TGACAACTGG
********** ********** ********** ***** ****  ********* **********
TGCAGGGATG AACAACATTT TATAAACATA GAGGATAACA GTCTCAGAAC TGACAACTGG

ACCGGACCTT TCAAGGATCA GTGGCATTTG CAACTAATTC AGTGCAAAAT TTTGGCGTTC
********** ********** ********** ********************** **********
ACCGGACCTT TCAAGGATCA GTGGCATTTG CAACTAATTC AGTGCAAAAT TTTGGCGTTC

TCTTCATCCA GCCATACTC
********** *********
TCTTCATCCA GCCATACTC
```

Figure 4.8c Sequence analysis of fragment F. Line one is the published nucleotide sequence of tPRL promoter (U05953) and line two is the nucleotide sequence obtained from the individual with F_2F_2 genotype. The base changes are in bold letters. Transcription factor binding motif with high similarity CdxA binding consensus sequences are in *italic and underlined.* The potential GATA2 binding motif is in ***bold, italic and underlined.***

The transcription factor binding motif search of this region revealed two putative transcription factor binding sites showing 90 to 96.8% similarity with known transcription factor binding motifs. The CdxA protein described in the previous section has 6 potential binding sites within fragment F while GATA2 has one transcription factor-binding motif (Fig. 4.8c). The transcription factor GATA2 is

another member of the zinc-finger family of transcription factors, which could act as a cofactor for Pit1 and plays a role in pituitary cell proliferation (Dasen *et al* 1999).

4.9 The PCR-SSCP analysis and characterisation of fragment G

Fragment G is a 327bp DNA fragment located at –239 to +88 of the tPRL promoter region. This segment of the tPRL gene promoter has been reported to contain a TATA box located at –27 from the transcription start site, a polyadenylation site, and two potential Pit1 binding sites (U05953). In a more recent study, Ohkubo *et al* (personal communication) indicated that functional Pit1 binding sites are present at the –123 to –98 region upstream of the chPRL gene. Initial investigation of fragment G included six SSCP conditions (Table 3.5), two of which (15% PAG at 8.5v/cm for 17h and 20% PAG at 8.5v/cm for 24h), showed a clear resolution of bands for 61 samples. Unlike the preceding polymorphic fragments, fragment G revealed two heterozygous (G_1G_2 and G_2G_3) and one homozygous genotype, G_1G_1. The migration pattern of the DNA single strands in a 15% PAG at 8.5v/cm for 17hr is depicted in Figure 4.9a.

The genotypic and allelic frequencies within the sample population for fragment G are listed in Table 4.9. It is interesting to note that the lowest group to exhibit broodiness consists of the G_1G_3 heterozygous individuals. The frequency of broody G_1G_3 hens was only 23% while the G_2 haplotype was observed to have the lowest frequency in the entire population. There were no homozygous individuals for G_2 or G_3 haplotype among the 61 birds studied. The allelic frequency analysis indicates that the presence of the G_2 haplotype increased the number of hens

expressing broodiness. Thus, the selection against broodiness during the development of the turkey strain in this study may have selected against the G_2 haplotypes decreasing their frequency in the current population.

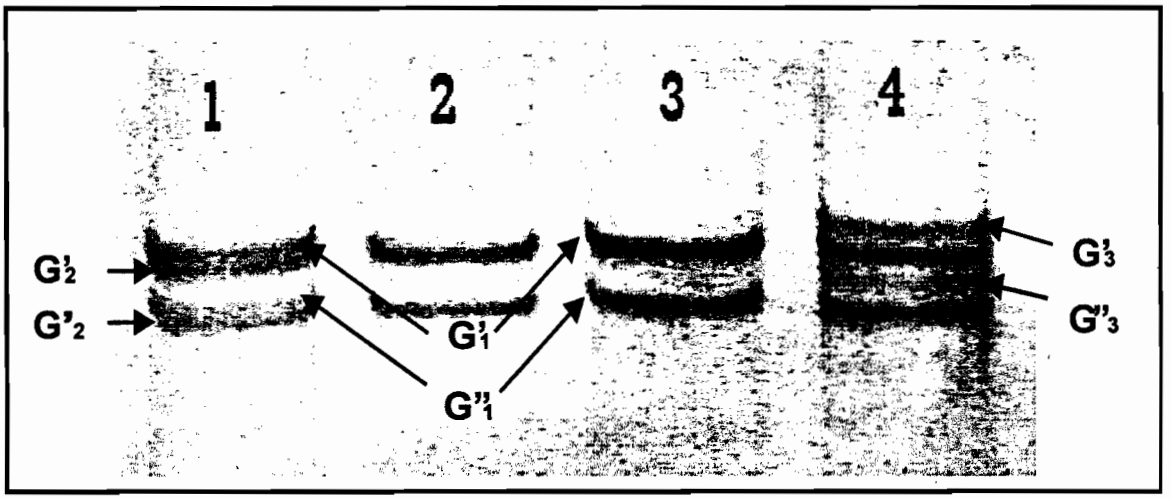


Figure 4.9a Detection of a three-allele SSCP in fragment G. The PCR amplified fragment G samples were separated in 15% PAG at 85v/cm for 17 h, at room temperature. Lanes 1 to 4 are genotypes G_1G_2, G_1G_1, G_1G_1 and G_1G_3 respectively.

Genotypes	Frequency (broody)	Frequency (nonbroody)	Frequency (population)	Total no. in population	Haplotypes	Frequency (haplotypes)
G_1G_1	0.26	0.74	0.44	27	G_1	0.721
G_1G_2	0.53	0.47	0.23	12	G_2	0.098
G_2G_2	-	-	-	-	G_3	0.180
G_1G_3	0.23	0.77	0.31	22		
G_3G_3	-	-	-	-		
				N=61		N=122

Table 4.9 The genotypic and allelic frequencies of fragment G polymorphism in the sample population.

The 61 birds comprising the sample population were genotyped. The genotypes were determined as G_1G_1 homozygote (n=27), G_1G_2 (n=12) and G_1G_3 (n=22) heterozygotes. The G_2G_2 and G_2G_3 homozygotes were not represented in the

sample population. Statistical analysis to test the genotypic effect on the levels of plasma PRL in the 61 individuals revealed that there was no association between the parameters tested.

Subsequent analysis tested the PRL response to genotype in subgroups based on physiological status. The birds were either incubating (n=18) or non-incubating hens (n=43), and the 18 incubating hens were either G_1G_1 homozygous (n=7), G_1G_2 heterozygous (n=6) or G_1G_3 heterozygous (n=5). In the non-incubating group 20 were G_1G_1 homozyous, 6 were G_1G_2 heterozygous, and 17 G_1G_3 were heterozygous individuals.

Analysis of variance was performed to test the genotypic effect on plasma levels of PRL in each of the following groups regardless of their physiological status. The analysis was first performed between the G_1G_1 (n=27) and the G_1G_2 (n=12) individuals, the second was performed between the G_1G_1 (n=27) and G_1G_3 (n=22) individuals, and the third was performed between the G_1G_2 (n=12) and G_1G_3 (n=22) individuals. Results from the three tests revealed that the G_1G_2 appears to be associated with the highest level of circulating PRL among the three genotypes. However, there was no a significant variation in the PRL response to the different genotypes ($p \geq 0.05$).

Analysis of variance also was used to test the genotypic effect on the levels of plasma PRL within the non-incubating subgroup. The analysis was first performed between the G_1G_1 (n=20) and the G_1G_2 (n=6) individuals, the second was performed between the G_1G_1 (n=20) and G_1G_3 (n=17) individuals, and the third was performed

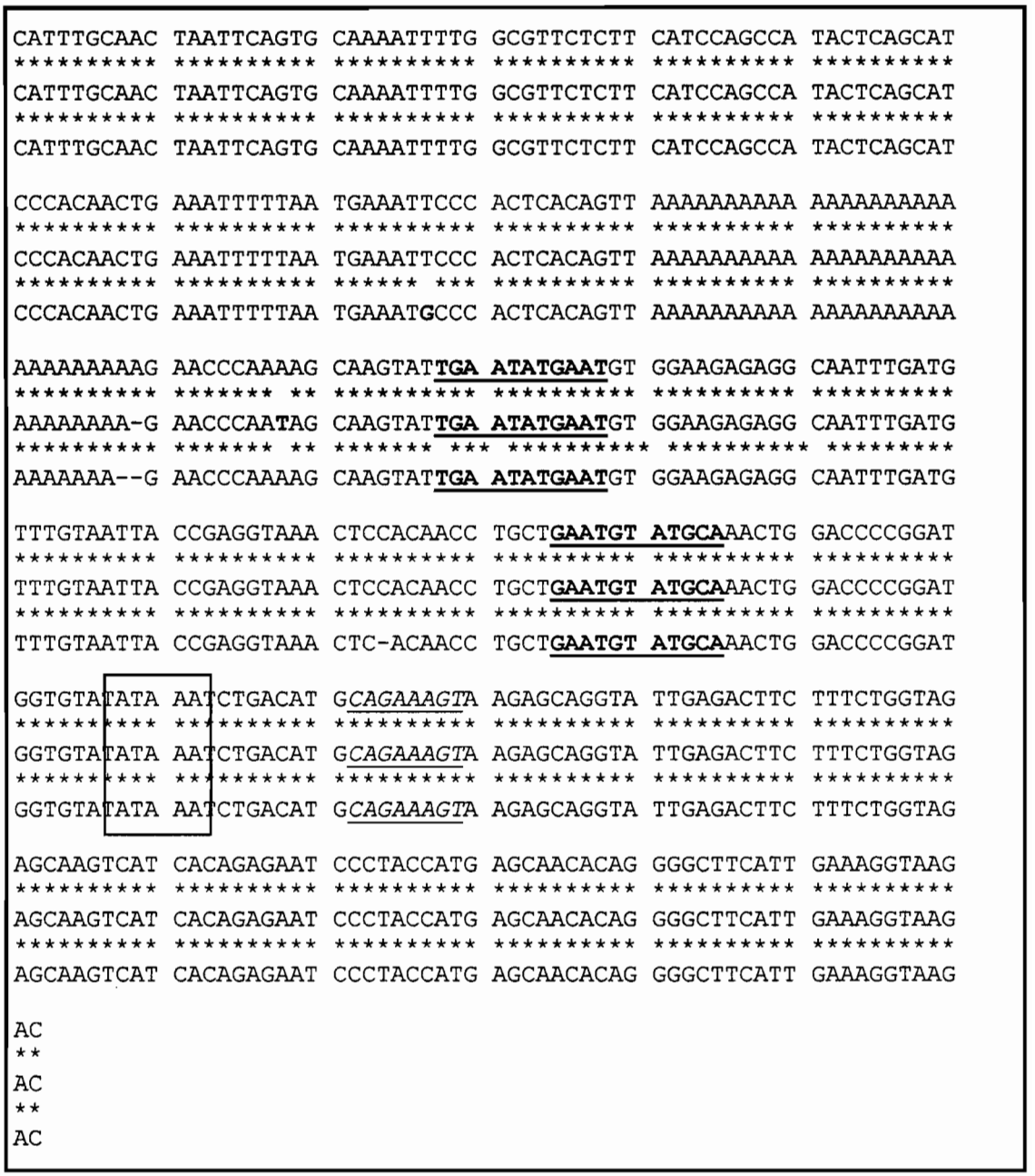


Figure 4.8b Sequence analysis of fragment G. First line represents the reported tPRL promoter sequence (U05953) and the nucleotide sequence of the G_1 haplotype. Lines two and three represent the nucleotide sequence of G_2 and G_3 haplotypes respectively. The putative Pit1 binding sites reported by Kurima *et al* 1995 are in ***bold and underlined***. The TATAA DNA motif is enclosed in a box and the 8bp core consensus sequence for avian and teleost Pit1 binding motif is in *italics and underlined*.

between the G_1G_2 (n=6) and G_1G_3 (n=17) individuals. All three results showed no association ($p \geq 0.05$) between the genotypes and the levels of plasma PRL.

Single factor analysis of variance was then performed to test the same parameters on individuals within incubating the subgroup. Seven of the incubating hens were G_1G_1 homozygous, 7 were G_1G_2 hetorozygous and 5 were a G_1G_3 heterozygous individual. The results showed that there was no association ($p \geq 0.05$) between the genotypes and levels of plasma PRL within the incubating subgroups.

The two heterozygotes (G_1G_2, and G_1G_3) and two G_1G_1 homozygotes were cloned and sequenced. The sequence results show that G_1 haplotype exhibited a nucleotide sequence identical to that of the reported tPRL promoter sequence (U05953) at that particular location while the G_2 haplotype has an A to T transition at the –137 position. The G_3 haplotype has a T to G transversion and a C deletion at –188 and –71 respectively (Fig 4.8b). In addition, G_1 haplotype as well as the published tPRL promoter (U05953) appears to contain a polyA rich section of 29 A's and G_2 has 28 A's in its polyA segment. The haplotype G_3 on the other hand, has 27 A's in its polyA segment.

Further analysis of fragment G shows a putative Pit1-binding motif on the antisense strand 7bp downstream of the TATA box (Fig 4.9b). To date however, this putative Pit1-binding site has not been investigated. In mammals, there has been no report of a functional Pit1-binding motif downstream of the TATA box thus this potential Pit1 binding consensus sequence may not be active. The position of this potential Pit1binding motif however, is interesting because when functional it could

act as an on/off switch for the transcription process. Binding of Pit1 in this region may disrupt PRL gene transcription by inhibiting formation of the transcription complex known to bind to the TATAA sequence to initiate the transcription process.

4.10. The PCR-SSCP analysis and characterisation of fragment H

Fragment H is a 188bp fragment located at –19 to 169 of the tPRL promoter and exon·1. The PCR-amplified fragment H was initially examined using four SSCP conditions (Table 3.5). The banding pattern was observed to be uniform in all samples investigated (Fig.4.10a). The SSCP result supports the earlier findings that this section of the PRL gene is highly conserved (Wong *et al* 1991). Sequence analysis of fragment H revealed the same nucleotide sequence as the reported tPRL sequence at this particular location.

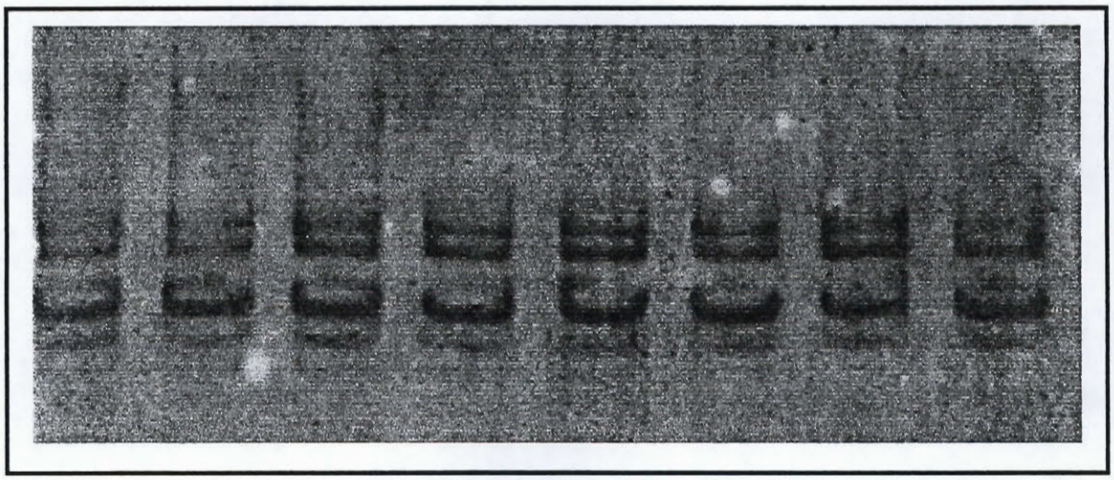


Figure 4.10a The PCR-SSCP analysis of fragment H. The PCR amplified fragment H samples were separated in 20% PAG at 8.5v/cm for 12h, at room temperature. No polymorphism was detected in this condition.

```
GACATGCAGA AAGTAAGAGC AGGTATTGAG ACTTCTTTCT GGTAGAGCAA GTCATCACAG
********** ********** ********** ********** ********** **********
GACATGCAGA AAGTAAGAGC AGGTATTGAG ACTTCTTTCT GGTAGAGCAA GTCATCACAG

AGAATCCCTA CCATGAGCAA CACAGGGGCT TCATTGAAAG GTAAGACTTT TGCTATTCCC
********** ********** ********** ********** ********** **********
AGAATCCCTA CCATGAGCAA CACAGGGGCT TCATTGAAAG GTAAGACTTT TGCTATTCCC

TGTCTGATAA CTTCTATGTT TAGGTTTTGA TTGAATTAAG AAGAAGCTGG AGGGTAACAA
********** ********** ********** ********** ********** **********
TGTCTGATAA CTTCTATGTT TAGGTTTTGA TTGAATTAAG AAGAAGCTGG AGGGTAACAA

TTCTAGAA
********
TTCTAGAA
```

Figure 4.10b Sequence analysis of fragment H. First line represents the reported tPRL promoter sequence at –19 to 169. No base sequence modification was detected when compared to sequence obtained in this study represented in the second line.

4.11 Summary of Findings

This study investigated sequence variations in the tPRL gene promoter region. The promoter region of eukaryotic genes have been demonstrated to regulate gene transcription via short DNA motifs that act as binding sites for *trans* and *cis*-acting transcription factors. To increase the SSCP sensitivity, the 2.4kb tPRL promoter was divided into nine sections designated as fragments A_d, to H with the size range of 188 to 370bp. Each fragment was subsequently ampliflied and examined. The SSCP analysis revealed that five (B, C, E, F and G) out of the nine fragments were polymorphic. The sample population made up of 62 birds was genotyped based on the mobility shift pattern of their single-stranded DNA. The hens were genotyped for each fragment and total of 302 genotypes were determined.

Blood samples from 62 birds kept in floor pens were collected at intervals predetermined as a function of age. Out of the 62 hens, 17 were observed to be incubating in nest boxes. The concentration of plasma PRL was determined by a homologous radioimmunoassay (Guemene *et al* 1994) of the blood samples collected at various stages during the 35-week turkey reproductive cycle. The association between the levels of plasma PRL and the genotypes were independently determined for each of the five polymorphic fragment because the genotypes were not completely linked. The analysis of variance was performed for each fragment to test the effect of genotype on the levels of plasma PRl regardlesss of turkey physiological status. The birds were subsequently divided in two groups based on their physiological status. Further statistical analyses were performed to test the genotypic effect on the levels of plasma PRL in the incubating and non-incubating birds.

Results from the statistical analysis showed that there was no conclusive association between the genotype and the levels of plasma PRL in 5 fragments investigated. This may be due to a low number of individuals in one genotype. When the 62 birds were placed into subgroups (incubating and non-incubating), the results show that in fragments B and C incubating subgroup there was an association between the parameters tested. However, the number of samples within the broody were low (n=17) and the genotype distribution is not balanced particularly in the C broody group (C_1C_1, n=12 and C_1C_2, n=5). The results from the analysis of the subgroups may not be adequate to form a conlcusion. The investigation of more samples is necessary for a more accurate conclusion.

Sequence analyses were performed to characterise the amplified fragments. A total of 13 polymorphic sites were identified. The location and characterisation of polynorphic sites are listed in Table 4.11. Further analysis revealed that three of the 13 polymorphisms were within DNA motifs of potential transcription factor binding sites. The C to T transition at –1402 altered the DNA sequence and the modified sequence is 91.4% similar to the human GATA1 binding motif (Joulin *et al* 1991). In mammals, GATA1 is involved in hematopeitic cell differentiation and may a role in the regulation of gene transcription (Dasen *et al* 1999). In avian species, GATA1 was also observed to play a role in hematopoietic cell differentiation but very few studies have been done to investigate the role of this transcription factor in the regulation of gene transcription.

The 12bp deletion at –1282 and the C insertion at –1246 were sequence variations that altered two Pit1 transcription binding sites. The 12bp deletion included an entire Pit1 binding motif while the C insetion altered the sequence of another potential Pit1 binding motif. Assays using chPRL promoter constructs with variable lenghts have shown that the chPRL promoter construct without the distal Pit1 binding sites had a higher transcription activity compared to those with the Pit1 binding motifs (Ohkubo *et al*, personal communication). On the other hand, studies in mammals have shown that point mutation within Pit1 binding sites in the PRL gene promoter reduce transcription activity (Iverson *et al* 1990). The studies imply that the Pit1 may play different regulatory roles in transcription of the mammalian and avian PRL gene. Figure 4.11 shows the location of several potential transcription factor response elements in the tPRL gene promoter.

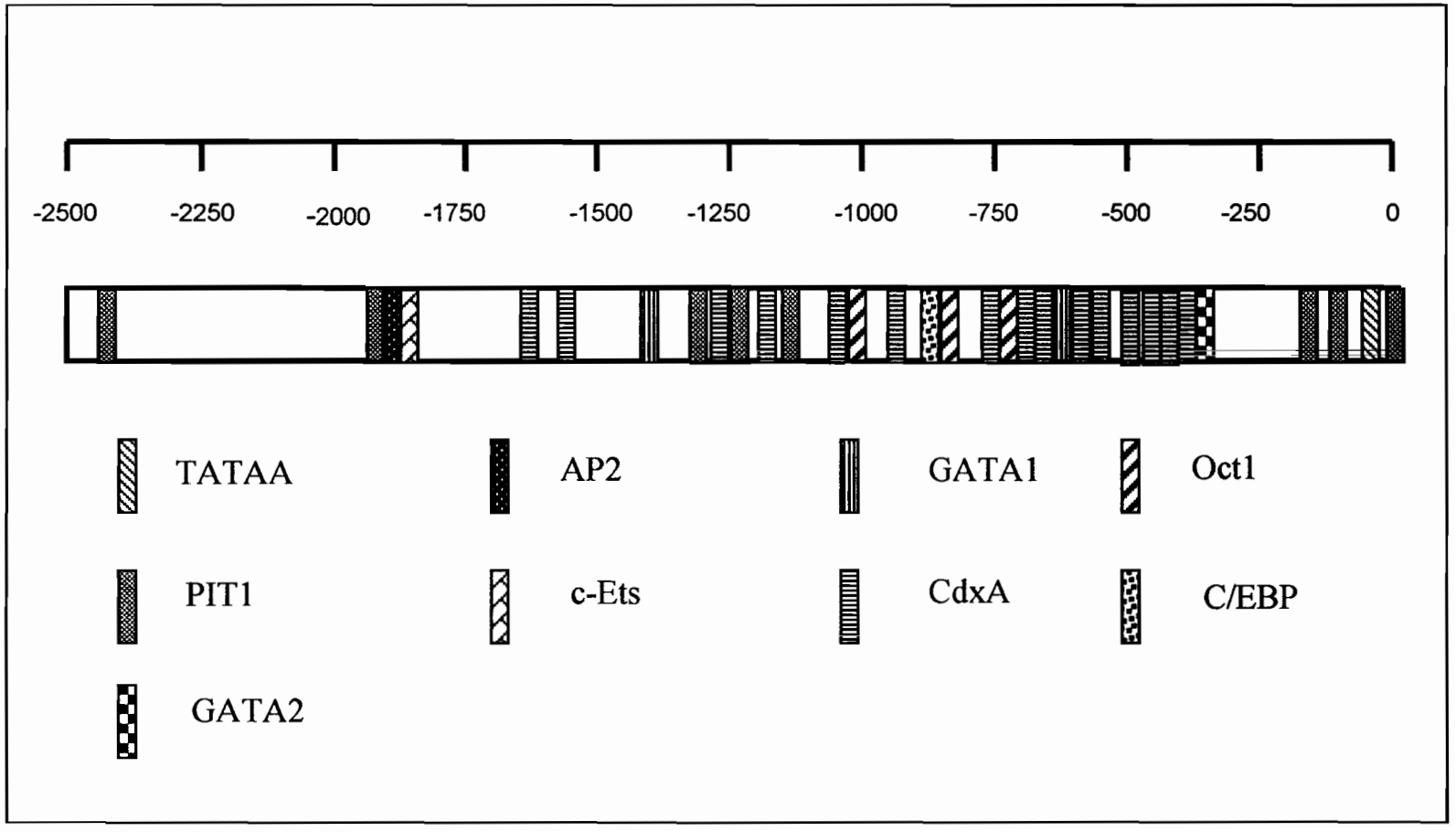
In turkey, the genotype having the 12bp deletion and C insertion appears to have higher levels of plasma PRL compared to those without the sequence variation. The result indicates that the Pit1 binding motif at the site of deletion played an inhibitory role in the transcription of the PRL gene. The C insertion in the other Pit1 binding consensus sequence altered the potential Pit1 binding motif. The modification of the consensus sequence may also alter the binding affinity of the DNA sequence to Pit1. The loss and inactivation of two Pit1 binding motifs may have a positive in the level of plasma PRL during the turkey reproductive cycle. The results support the inhibitory action of Pit1 on the avian PRL gene transcription. However, the presence of Pit1 binding sites in the chicken and turkey PRL gene does not necessarily indicate a transcriptional role because this transcription factor has a definitive role in pituitary cell differentiation.

In addition to Pit1, the tPRL promoter contains several short DNA motifs with more than 90% similarity to known transcription factor binding consensus sequences. These short DNA sequences include binding sites for AP1 or AP2, c-Ets, Oct1, C/EBP, GATA1 and GATA2, and the 7bp DNA motif that binds to CdxA. All these transcription factors except CdxA has been demonstrated to play a role in the formation of transcription factor complexes and regulates PRL gene expression in mammals. Some of these transcription factors particularly Oct1, and c-Ets are involved in pituitary cell differentiation and proliferation also observed in mammals. It appears that selection against broodiness by turkey breeders has decreased the number of turkey with the likelihood of expressing an elevated plasma PRL level and consequent expression of this maternal behaviour.

Fragments	Polymorphism	Location
B	C to T transition	-1402
	T to C transition	-1400
C	12bp deletion	-1282 to -1271
	C insertion	-1246
	A to G transition	-1206
	T to C transition	-1184
E	A to G transition	-728
	T to C transition	-654
F	G to T transversion	-323
	A to G transition	-319
G	T to G transversion	-188
	A to T transversion	-137
	C deletion	-71

Table 4.11 Summary of polymorphisms in the tPRL gene promoter.

Figure 4.11 Location of potential transcription factor response elements



CHAPTER V. CONCLUSION

The SSCP analysis detected variants in 5 out of the nine fragments covering the 2.4kb of the tPRL gene promoter. Sequence analysis of the fragments revealed 13 polymorphic sites. Three out of the 13 identified appeared to be associated with the levels of plasma PRL during a 35-week period of the turkey reproductive cycle. The three sites were identified as a T to C transition at –1400, a 12bp deletion at –1282 to –1271 and a C insertion at –1246. The C to T transition altered the sequence at the particular region and the modified sequence matched that of the GATA1 transcription factor binding DNA motif. The 12bp deletion at –1282 to –1272 was determined to contain a putative Pit1 binding site and the C insertion at –1246 altered the sequence of another putative Pit1 binding motif. The individuals with all three polymorphic sites appear to have a 50% chance of becoming broody.

The significant effect of genetic variants on the 35-week level of plasma PRL implies that these DNA markers may be used for selecting against the expression of incubation behaviour in commercial flocks. Indeed, the observation that certain genotypes and genotypic combinations occur at relatively low frequency within the population suggests traditional selection strategies may have already selected against these genetic variants. Artificial selection in the commercial turkey industry may have reduced the frequency of these allotypes in concert with a reduction in the incidence of broodiness in breeder hens.

Further studies using a larger number of animals are required to investigate the inter-relationship between genotypes, hyperprolactinemia and the expression of

broodiness in turkey hens. This study has shown that some of these genetic variants affect the binding of several transcription factors which are thought to regulate the transcription of PRL gene as well as controlling pituitary cell differentiation. Additional experiments using reporter genes or gel mobility shift assay in concert with the various allelic variants detected in the promoter may clarify the effects of altered binding domains on rates of transcription.

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