Evidence for low homology between mammalian leptin and chicken leptin-like gene sequences.

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DECLARATION

I hereby declare that this thesis has been composed by myself and has not been submitted for any other degree elsewhere. The work presented herein is my own, and all assistance to me is acknowledged.

GODAVARMA GIRISH VARMA.

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ABBREVIATIONS

A	adenosine
ACTH	adrenocorticotropic hormone
AGRP	agouti-related peptide
AMP	adenosine mono phosphate
Arg	arginine
ATP	adenosine tri phosphate
BAC	bacterial artificial chromosome
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
cAMP	cyclic Adenosine mono phosphate
CART	cocaine- and amphetamine-regulated transcript
CCK	cholecystokinin
cDNA	complementary deoxyribonucleic acid
C/EBP	CAAT/enhancer binding protein
CNS	central nervous system
cpm	counts per minute
CRF	corticotropin releasing hormone
C-terminal	carboxy terminal
CTP	cytosine tri phosphate
DMSO	dimethylsulfoxane
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
E.coli	Escherichia coli
EDTA	ethylenediaminotetra-acetic acid
fa	fatty
FSH	follicle stimulating hormone
g	gram(s)
G	guanosine
GAPDH	glyceraldehyde phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
GH	growth hormone
GLP-1	glucagon-like peptide-1
GTP	guanine tri phosphate
GnRH	gonadotropin releasing hormone
ICC	immunocytochemistry
icv	intracerebroventricular
IGF-1	insulin-like growth factor-I
IL-1	interleukin-1
IPTG	isopropylthio-β-D-galactoside
JAK	Janus kinase
kb	kilo base(s)
LEP-R	leptin receptor
M	mole(s)
MBq	mega Becquerel
MC-4	melanocortin-4

min	minute(s)
ml	milli litre(s)
mM	milli mole(s)
mRNA	messenger ribonucleic acid
MSH	melanocyte stimulating hormone
N-terminal	amino terminal
ng	nano gram(s)
nM	nano mole(s)
NPY	neuropeptide Y
OB	obese
OD	optical density
OLB	oligo labelling buffer
pBSK	Blue skript plasmid
PCR	polymerase chain reaction
pM	pico mole(s)
POMC	proopiomelanocortin
PVN	paraventricular nucleus
QTL	quantitative trait locus
RNA	ribonucleic acid
RNAse	ribonuclase
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
s	second(s)
SDS	sodium dodecyl sulphate
SSC	standard sodium citrate
STAT	signal transducers and activators of transcription
T	thymine
TAE	Tris acetate electrophoresis buffer
TBE	Tris borate electrophoresis buffer
TBq	tega Becquerel
TE	Tris EDTA
TEMED	tetramethyl ethylenediamine
Trp	tryptophane
UV	ultra violet
XGAL	5-Bromo-4chloro-3-inodyl-β-D-galactoside
YAC	yeast artificial chromosome
μg	micro gram(s)
μl	micro litre(s)
μm	micro mole(s)

ABSTRACT

Leptin is a 167-amino acid hormone produced chiefly by adipocytes. It plays an important role in regulation of food intake, energy metabolism and reproduction in mammals. However, a leptin gene homologue has yet to be cloned in a non-mammalian vertebrate. The aim of this thesis was to establish the existence of a leptin gene homologue in the domestic chicken (Gallus gallus) genome, and to determine the degree of sequence identity with mammalian leptin genes, and with a putative chicken leptin sequence published during the course of the thesis work. An initial attempt was made to clone the chicken leptin gene by heterologous RT-PCR using degenerate primers to conserved regions of mammalian leptin sequences. However, no leptin-like products were amplified from chicken adipose tissue and liver cDNAs, or from genomic DNA. RT-PCR was also used to test the existence of a published chicken leptin cDNA sequence that shares 95% identity with mouse leptin at the nucleotide level. When PCR primers identical to the mouse and published chicken leptin sequences were used, no PCR product sharing close similarity to the mouse leptin sequence were generated from any chicken templates, whereas amplification of mouse leptin leptin sequences was consistently obtained from control mouse templates. Following the failure to clone the chicken leptin by RT-PCR, evidence for the existence of a mammalian-like leptin in the chicken genome was sought by Southern analysis. Southern blots under low stringency hybridization and washing conditions revealed hybridization of a mouse leptin probe to chicken genomic DNA. With high stringency washing, the chicken signal disappeared, while those from sheep and mouse genomic DNA remained. Screening of a chicken adipose tissue cDNA library, and chicken genomic DNA and cosmid libraries with the same mouse probe failed to isolate a chicken leptin homologue. Collectively, these results indicate that if a chicken leptin homologue exists in the chicken genome, it is likely to be of low homology to mammalian leptin sequences. The results do not support the existence of a mouse-like leptin sequence in the chicken genome, an assertion supported by theoretical analysis of the molecular evolution of leptin based on the rate of synonymous substitution. This analysis indicated that the probability that the chicken and mouse leptin sequences are 95% identical, is less than one in a million.

CHAPTER 1

INTRODUCTION

1.1 General background

The dominant paradigm in biological science, in general, and in the control of food intake in particular, is fundamentally homeostatic (Rowland *et al.* 1996). The assimilation, storage, and disposition of nutrient energy constitute a complete homeostatic system, which is central to the survival of both prokaryotic and eukaryotic organisms. In vertebrates, storage of energy-dense fuel in the form of adipose tissue triglyceride permits survival during prolonged periods of food deprivation (Friedman 1997). In order to maintain such fuel stores during times of nutritional scarcity or surplus, a balance between energy intake and expenditure must be achieved. Changes in energy balance are reflected in the size of the fat stores. White adipose tissue is the principal energy buffer, and the main variable in overall body composition. The regulation of energy balance is, therefore, frequently considered as being synonymous with the control of body fat (Trayhurn and Rayner 1996).

Leptin is a protein hormone synthesized chiefly by white adipose tissue, and its discovery has opened up a new area of research into mechanisms controlling obesity (Campfield *et al.* 1996). This is one of the most prevalent nutritional/metabolic disorders in developed and developing nations (Zimmet and Collier 1996). Research on leptin started with the positional cloning of the mouse obese (leptin) gene in 1994 by Jeffery Friedman and co-workers (Zhang *et al.* 1994). The polypeptide hormone product of the leptin gene (after the Greek term *leptos*, meaning thin) was shown to act as a peripheral satiety signal to the brain in the regulation of energy homeostasis (Flier 1997). Subsequently, leptin has been shown to influence many body functions including feeding, thermogenesis, reproduction,

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immune responsiveness, haemopoiesis, angiogenesis and development (reviewed by Friedman 1997; Houseknecht *et al.* 1998; Friedman and Halaas 1998; Hossner 1998).

1.2 Historical background

The principal factors responsible for the regulation of body weight and energy balance in mammals have been the subject of extensive investigation. The first clue pointing to the involvement of the brain in body weight regulation was provided by patients with Frohlich's syndrome, who have tumours of the basal hypothalamus, and develop massive obesity (reviewed in Rowland *et al.* 1996). Hetherington and Ranson (1939) were able to reproduce this syndrome with experimentally produced lesions (Brobeck *et al.* 1943). Using a parabiosis (cross-circulation) experimental design G.R.Hervey first demonstrated the presence of agents in blood that regulate body weight by providing a satiety signal to the hypothalamus (Hervey 1958). Kennedy (1953) noted that body fat accounted for the most of the usable stored fuels in mammals. He suggested, in his lipostatic theory of body weight control, that adipose tissue might produce a hormone providing a signal to the brain to regulate food intake. In contrast, Mayer (1955) emphasized in his glucostatic hypothesis; the importance of blood glucose as a fuel and satiety signal to the brain.

Important information on the genetic control of body weight and energy balance came from the discovery by Ingalls and colleagues (Ingalls *et al.* 1950) of a recessive mutant in mice associated with massive obesity. The genetic defect in the obese or *ob/ob* mouse is a recessively inherited trait, manifested early in life, associated with diabetes. Later, a second form of recessively inherited obesity in the diabetes or *db/db* mouse was described by Coleman (1978).

Recessive mutations in the mouse *obese* (leptin) and *diabetes* (*db*) genes result in obesity and diabetes in a syndrome resembling morbid human obesity. The *ob/ob* and *db/db* mice have identical phenotypes, each weighing three times more than normal mice (even when fed the same diet) and have a five fold greater body fat content (Friedman and Halaas

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1998). Data from parabiosis experiments suggested that the *ob* gene encoded, or was responsible for the generation of, a circulating factor that regulated energy balance, and that the *db* gene encoded the receptor. These data, when considered in conjunction with the results of parabiosis experiments (Hervey 1958) using rats with hypothalamic lesions, further suggested that the *ob* receptor was localized in the hypothalamus. From parabiosis experiments between *ob/ob*, *db/db* and normal (wild type) mice, Coleman concluded that *ob/ob* mice do not produce a circulating satiety factor and that *db/db* mice overproduce the factor, but lack its receptor (Coleman 1978). The cloning of the *ob* (Zhang *et al.* 1994) and *db* (Tartaglia *et al.* 1995; Chen *et al.* 1996a) genes, and observations on their expression in body tissues, show that body fat content is regulated by a negative feedback loop involving leptin, the protein product of the *ob* gene produced in adipose tissue, and leptin receptor, the protein product of the *db* gene, expressed in the hypothalamus.

1.3 Positional cloning of leptin gene

In initial studies, the *ob* gene was mapped to mouse chromosome 6 close to a restriction-fragment length polymorphism (RFLP) marker, D6Rck13, identified from chromosome microdissection (Friedman 1991; Bahary *et al.* 1993). It was also found that Pax4 in the proximal region of mouse chromosome 6 was tightly linked to *ob* (Walther *et al.* 1991). Both loci were initially used to type a total of 835 informative meioses derived from both interspecific and intersubspecific mouse crosses that segregated for leptin. Pax4 was mapped proximal to *ob* and no recombination between D6Rck13 and *ob* was detected among the 835 meioses (Bahary *et al.* 1993).

To position *ob* more precisely, an additional 771 meioses derived from both a C57BL/6J *ob/ob* X DBA/2J intercross and backcross were genotyped. In this way the genomic position of the gene was identified within centimorgans. In order to construct a physical map in the region of *ob*, the DNA in the region of Pax4 and D6Rck13 markers was cloned in yeast artificial chromosomes (YACs) and the clones were screened using probes for both of the markers. Telomeric and centromeric ends of the YAC clones were recovered and used for further screening until clones mapping to the *ob* locus were

obtained. DNA from individual or pools of clones was subcloned into the pSPL3 exon trapping vector (Church *et al.* 1994) and each exon trapping product was sequenced and compared to all sequences in Genbank. One of the trapped exons was hybridized to a Northern blot of mouse tissues and detected a ~4.5kb mRNA sequence derived from white adipose tissue. This was confirmed using the reverse transcribed polymerase chain reaction (RT-PCR) technique.

1.4 The Leptin gene

After they were cloned, the *ob* gene was referred to as the leptin gene, and the *db* gene as the leptin receptor gene or LEP-R. The mouse leptin gene consists of 3 exons and 2 introns (figures 1.1, 1.2) and encodes ~4.5kb of mRNA (Zhang *et al.* 1994). It maps to the proximal end of chromosome 6 (Friedman 1991; Bahary *et al.* 1993). The genomic and promoter organization of the mouse leptin gene is shown in figure 1.1. A single TATA- containing promoter, supposedly being a target of CAAT/enhancer binding protein (C/EBP), is found upstream of exon 1. The 161 bp minimal promoter contains consensus Sp1 and CCAAT/enhancer- binding protein (C/EBP) motifs. Co-transfection with C/EBPα, a transcription factor important in adipose cell differentiation, causes a 23-fold activation suggesting that the leptin promoter is a natural target for C/EBPα (He *et al.* 1995).

Southern blot analysis demonstrates a single copy of the leptin gene in the human genome spanning ~20kb containing 3 exons separated by 2 introns (Isse *et al.* 1995). The first intron (10.6kb) occurs in the 5'- untranslated region, 29 bp upstream of the ATG start codon and the second intron (2.3 kb) at glutamine +49. The transcription initiation site is 54-57 bp upstream of the ATG start codon. The 172 bp 5'-flanking region contains a TATA box-like sequence and several cis-acting regulatory elements (3 copies of GC boxes, an Ap-2 binding site, and a C/EBP binding site). The gene is assigned to human chromosome 7q31.3 (Isse *et al.* 1995). A functional analysis of the promoter demonstrates that only 217 bp of the 5' sequence is required for basal adipose tissue-

Sp1   C/EBP   TATA Box

Exon 1

GGGATCCCTG CTCCAGCAGC TGCAAG

481 bases

Alternate Exon 1

GCTCTGC TGCAATAGTC CCCAGACGTG AGGAACACAG TGTACTGCAT CAGTATTTTA GCAATTCCTC TGCCAGCTCC

GAGGCTATAA CAACTC

Intron 1   3.3kb

EXON 2

GTGCAAGAAG AAGAAGATCC CAGGGAGGAA AATGTGCTGG AGACCCCTGT GTCGGTTCCT GTGGCTTTGG TCCTATCTGT
CTTATGTTCA AGCAGTGCCT ATCCAGAAAG TCCAGGATGA CACCAAAACC CTCATCAAGA CCATTGTCAC CAGGATCAAT
GACATTTCAC ACACG

Intron 2

EXON 3

CAGTC GGTATCCGCC AAGCAGAGGG TCACTGGCTT GGACTTCATT CCTGGGCTTC ACCCCATTCT GAGTTTGTCC
AAGATGGACC AGACTCTGGC AGTCTATCAA CAGGTCCTCA CCAGCCTGCC TTCCCAAAAT GTGCTGCAGA TAGCCAATGA
CCTGGAGAAT CTCCGAGACC TCCTCCATCT GCTGGCCTTC TCCAAGAGCT GCTCCCTGCC TCAGACCAGT GGCCTGCAGA
AGCCAGAGAG CCTGGATGGC GTCCTGGAAG CCTCACTCTA CTCCACAGAG GTGGTGGCTT TGAGCAGGCT GCAGGGCTCT
CTGCAGGACA TTCTTCAACA GTTGGATGTT AGCCCTGAAT GCTG

Figure 1.1. Nucleotide sequence of the mouse leptin gene. The gene has 3 exons (bases shown in upper case) and 2 introns (bases shown in lower case). Leptin is encoded within exon 2 and 3 (shown in red). The GGATCC start site in the Exon 1 is underlined. Parts of the upstream nucleotide sequence (-953 bases) of the gene also is shown containing putative Sp1, C/EBP, and TATA sequences (underlined and labelled). [Data from He *et al.* 1995]

specific expression of the leptin gene and for enhanced expression by C/EBPα (Miller *et al.* 1996).

Mutations in the leptin gene (figure 1.2) that prevent the synthesis of leptin in two strains of *ob/ob* mice have been identified (Moon and Friedman 1997). In the C57BL/6J *ob/ob*[1J] (figure 1.2, panel b) mutant, a nonsense mutation (single-base mutation in codon 105) results in the replacement of arginine by a premature stop codon. This results in synthesis of a truncated protein that is degraded in the adipocyte (Zhang *et al.* 1994). In SM/Ckc-+[Dac]*ob*[2J]/*ob*[2J] mutation (figure 1.2, panel c), a ~5-kb Etn transposon is inserted into the first intron of the leptin gene. This mutation results in the synthesis of hybrid mRNAs in which the splice donor of the non-coding leptin first exon is joined to splice acceptors in the transposon. Mature leptin mRNA is not synthesized in this mutant. In another mutation, no leptin mRNA is detectable in the adipose tissue because of a polymorphism in the promotor region of the gene, which is hypothesized to interfere with transcription (Friedman and Halaas 1998).

In contrast to *ob/ob* mice, obese human subjects usually synthesize some leptin. DNA screening studies of obese human populations have ruled out the possibility that a mutation similar to mouse *ob/ob* is a common cause of obesity in man (Considine *et al.* 1995; Maffei *et al.* 1996). Nevertheless, a few leptin gene mutations have been described in humans. For example, polymorphism at the leptin locus has been reported to be associated with extreme obesity (Considine *et al.* 1996a) where two cousins who were homozygous for a frameshift mutation in the leptin gene were markedly obese and did not have any circulating leptin (Montague *et al.* 1997). Further three members of a Turkish family who were grossly obese were found to have a missense mutation in the leptin gene similar to that in C57BL/6J *ob/ob* mice (figure 1.2b) (Strobel *et al.* 1998). In mice, this mutation generates a stop-codon; in humans it substitutes Arg-105 with Trp. The mutant human leptin cannot be secreted by the cells because it is degraded in the adipocyte (Zhang *et al.* 1994).
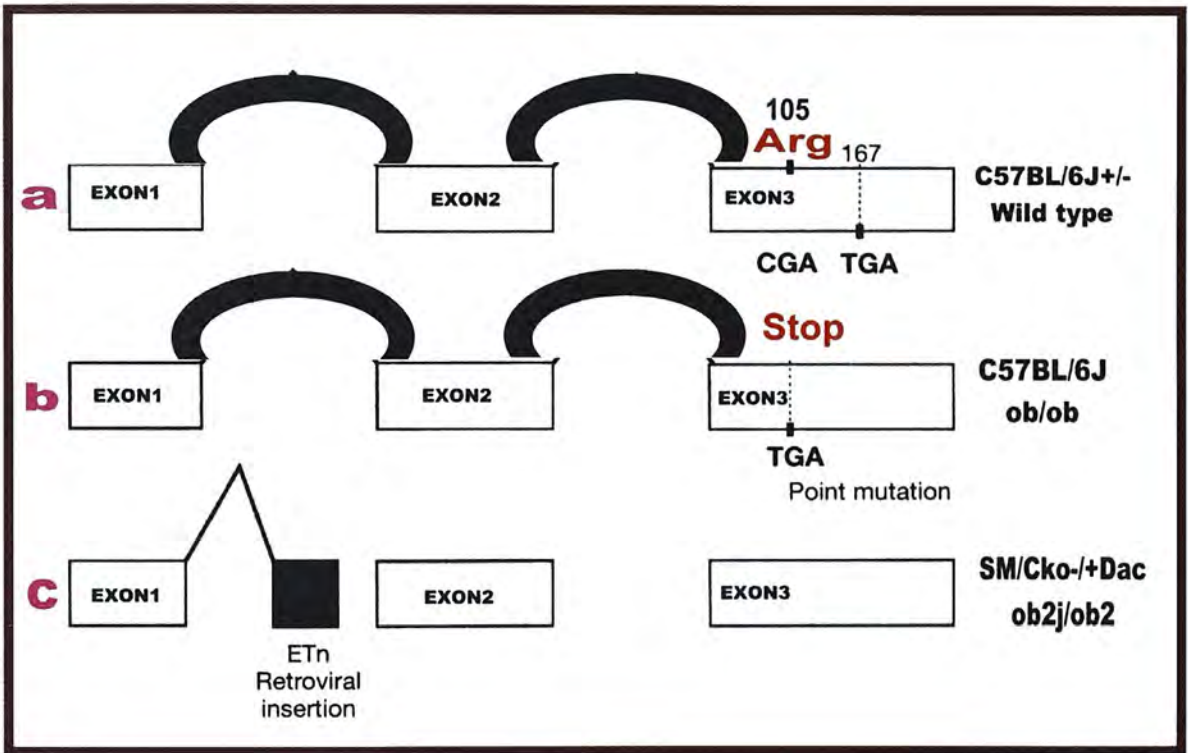
Figure 1.2. Molecular basis of ob mutations in mouse leptin gene. The wild genotype of the mouse (a) produces an intact 167 amino acid leptin while in the C57BL/6J ob/ob obese mouse (b), a non-sense mutation at codon 105 creates a TGA stop codon, resulting in the synthesis of a truncated protein that is inactive. In SM/Cko-/+Dac ob^{2j}/ob^{2j} obese mouse (c) Etn retroviral insertion in intron 1 (transposon) results in abnormal splicing of the leptin gene such that a hybrid mRNA is created which includes retroviral RNA and mature leptin RNA is not expressed (modified from Friedman 1997).

1.5 Leptin gene expression

The leptin gene is expressed in white adipose tissue, as shown using *in situ* hybridization and immunohistochemistry (Maffei *et al.* 1995). Some studies on brown adipose tissue report little or no expression of the leptin gene (Maffei *et al.* 1995; Trayhurn *et al.* 1995a), while others report high levels of leptin mRNA (Moinat *et al.* 1995). This discrepancy may be because it is difficult to demonstrate leptin mRNA by Northern blotting. However, leptin gene expression is demonstrated when the more sensitive technique of reverse transcription polymerase chain reaction (RT-PCR) is employed (Trayhurn *et al.* 1997). Leptin gene expression in brown adipocytes has also been demonstrated using immunocytochemistry in *db/db* mice (Cinti *et al.* 1997) and in primary culture (Deng *et al.* 1997). Leptin gene expression has also been demonstrated in brown fat in the Djungarian hamster (Klingenspor *et al.* 1996).

The leptin gene is expressed at low levels in the gastric epithelium (Bado *et al.* 1998), skeletal muscle (Wang *et al.* 1998), and in the brain and pituitary gland (Morash *et al.* 1999). The leptin gene is also expressed in placenta (Green *et al.* 1995; Hoggard *et al.* 1997; Masuzaki *et al.* 1997; Ashworth *et al.* 2000) where it may play a role in the signalling of maternal-foetal energy reserves. These observations demonstrate that the functions of leptin are not restricted to the regulation of fat deposition.

Physiological and hormonal factors, which affect the expression of the leptin gene and the secretion of leptin, have been identified from studies *in vivo*. Physiological factors include fasting, cold exposure, and exercise (Trayhurn 1995a and b; Hardie *et al.* 1996b; Zheng *et al.* 1996). All result in decreased leptin gene expression and a reduction in the circulating level of leptin. In fasted subjects, subsequent refeeding leads to a rapid restoration of leptin gene expression and leptin secretion (Becker *et al.* 1995; Saladin *et al.* 1995; Trayhurn *et al.* 1995b; Hardie *et al.* 1996b). Trayhurn *et al.* (1995b) showed that overnight fasting provokes a significant decrease in leptin mRNA in mouse adipose tissue. The same effect was observed in rats by Saladin and colleagues (1995). Conversely, after an overnight fast in rats, rapid feeding is associated with an increase in

leptin mRNA in adipose tissue. These observations provide compelling evidence that, in rodents, leptin accumulates in the plasma up to a point where satiety occurs, resulting in reduced consumption of food followed by a drop in leptin secretion and leptin gene expression in adipose tissue (Saladin *et al.* 1996).

Glucocorticoids and insulin stimulate leptin gene expression (Becker *et al.* 1995; Devos *et al.* 1995; Saladin *et al.* 1995). A time-dependent effect of glucocorticoids on the induction of leptin mRNA, with a maximum effect after two days of treatment, is observed following a subcutaneous injection of hydrocortisone, triamcinolone or dexamethasone (De Vos *et al.* 1995). Observations on primary cultures of adipocytes (Murakami *et al.* 1995) suggest that glucocorticoids act directly to induce leptin gene expression. This conclusion is supported by the identification of several glucocorticoid response element consensus binding sites (GREs) in the proximal promoter of the gene (Gong *et al.* 1996). Additional support for these effects is provided by the earlier observation, that glucocorticoid injection in *ob/ob* mice does not result in decreased feeding or body weight, as it does in normal animals (McGinnis *et al.* 1987). Presumably this is because *ob/ob* mice do not make functional leptin.

Insulin in particular, is viewed as a key regulator of leptin gene expression. Insulin injection mimics the effect of feeding in stimulating leptin gene expression in adipose tissue (Saladin *et al.* 1996). Conversely, streptozotocin, a blocker of insulin secretion, reduces adipose tissue leptin mRNA levels, which are restored after insulin treatment (MacDougald *et al.* 1995). This effect is caused by insulin, as opposed to glucose, since both hyper- and euglycaemic hyperinsulinaemic clamps result in the same effect on adipose tissue leptin mRNA (MacDougald *et al.* 1995). The effect of insulin appears to be direct since leptin mRNA levels increase after insulin treatment in primary cultures of adipocytes (Saladin *et al.* 1995) and in an adipocyte cell line (differentiated 3R3-L1 cells, MacDougald *et al.* 1995). These observations explain why all hyperinsulinaemic animal models of diabetes and obesity are characterized by high leptin mRNA levels in adipose tissue (Ogawa *et al.* 1995; Trayhurn *et al.* 1995b; Maffei *et al.* 1995).

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In contrast to the stimulatory effects of insulin, noradrenaline and the non-specific β-adrenoceptor agonist, isoproterenol, inhibit leptin gene expression (Trayhurn et al. 1995a). These effects occur primarily through β3-adrenoceptors, since selective β3-agonists have a powerful inhibitory effect on leptin gene expression and rapidly reduce circulating leptin levels (Mantzoros et al. 1996; Trayhurn et al. 1996). These observations suggest that the sympathetic nervous system exerts an inhibitory feedback action on white adipose tissue, regulating leptin production by inhibiting leptin gene transcription through the β3-adrenoceptor subtype (Trayhurn et al. 1996). If this is the case, then during a fast, a decrease in serum insulin may not be the cause of the reduction in leptin gene expression and circulating leptin. The critical factor may be the inhibitory effects of an increased sympathetic drive to white adipose tissue.

Leptin is thought to close a short inhibitory feedback loop controlling its own synthesis in adipose tissue. This is suggested by a study in Zucker rat pups with different copy numbers of the leptin receptor mutation, LEP-R[fa] (fa, Zhang et al. 1997a). In these animals the concentration of plasma leptin is directly related to copy number of the mutation (fa/fa > fa/+ > +/+). These relationships are independent of fat mass and the concentration of serum insulin. Reduced copy number of functional leptin receptor in adipose tissue results in a diminished negative feedback signal to the leptin gene and an increased expression. The absence of overt obesity and insulin resistance in these rat pups is consistent with this view.

Other modulators of leptin gene expression have also been identified. For instance, the level of adipose leptin mRNA decreases after treatment with cyclic AMP (Slieker et al. 1996), β3 adrenergic-receptor agonists (Moinat et al. 1995; Slieker et al. 1996), and antidiabetic thiazolidinediones (De Vos et al. 1996). Endotoxins and cytokines, compounds well known to mediate the inflammatory host response to infections, and important regulators of adipose tissue metabolism, stimulate leptin gene expression in hamsters (Grunfield et al. 1996). These data are especially relevant since induction of leptin gene expression during host defence may contribute to the anorexia associated with infection.

Less is known in humans about the regulation of leptin gene expression since changes in expression have not been reported (Saladin *et al.* 1996). However, leptin gene expression in human adipose tissue is positively correlated with adipocyte hypertrophy and hyperplasia and adipose tissue volume (Hamilton *et al.* 1995). Insulin clamp studies are consistent with a slow-acting stimulatory effect of insulin on leptin gene expression (Pagano *et al.* 1997). Further, insulin up-regulation of leptin gene expression is seen in primary cultures of human adipocytes transfected with plasmids containing the 5'-flanking sequences of the rat leptin gene fused to the luciferase gene (Fukuda and Iritani 1999). It has also shown that -101 to −83 (5' region) of the human leptin gene is responsible for glucose/insulin stimulation of transcription (Fukuda and Iritani 1999).

1.6 Physical structure of leptin

The cDNA sequence of the leptin gene (Zhang *et al.* 1994; Murakami and Shima 1995; Hotta *et al.* 1996; Bidwell *et al.* 1997; Dyer *et al.* 1997; Ji *et al.* 1998; Walder *et al.* 1997) predicts a 167-amino acid protein with a molecular mass of 16,024 Da. This has been confirmed by characterizing naturally occurring leptin in blood (Halaas *et al.* 1995; Cohen *et al.* 1996a). When endogenous leptin is isolated from serum from moderately obese women using immunoaffinity chromatography and SDS-PAGE chromatography, the principal band shows the same mobility as recombinant- derived human leptin, with a molecular mass of 16,000Da (Cohen *et al.* 1996a). No evidence for post-translational modifications in endogenous leptin has been obtained (Cohen *et al.* 1996b). A 19K form of leptin has been identified in extracts of rat stomach, but the molecular structure and functional significance of this form is not known (Bado *et al.* 1998).

X-ray crystallography cannot be carried out on leptin directly because it aggregates extensively, which inhibits crystallization. However, a single amino acid substitution of Glu for Trp at position 100 in leptin results in a protein, termed $Glu^{100}OB$ or leptin-E100. Compared with native leptin, leptin E100 has increased surface hydrophobicity and comparable biologic activity but has dramatically improved solubility and a propensity to

crystallize (Zhang *et al.* 1997a and b). The structure of leptin-E100 molecule is shown in figure 1.3. The protein is an elongated molecule with approximate dimensions of 20 Å % 25 Å % 45 Å. It consists of four antiparallel α-helices (A, B, C, and D), connected by two long crossover links and one short loop, and arranged in a left-hand twisted helical bundle. Leptin contains two cysteine residues (at 96 and 146), which form a disulphide bond between the C-terminus of the protein and the beginning of the CD loop (figure 1.3). Mutation of either of these conserved cysteines result in a biologically inactive protein (Zhang *et al.* 1997b). This indicates that disulphide formation and the subsequent kinked D-helix structure are important for protein folding and/or receptor binding.

A comparison of the crystal structures of leptin-E100 (Zhang *et al.* 1997a), leukaemia inhibiting factor (LIF) and ciliary neurotropic factor (CNTF) (Robinson *et al.* 1994; McDonald *et al.* 1995) suggest that leptin has two potential receptor-binding sites. A receptor-binding model for leptin-leptin-receptor (LEP-R) has been proposed similar to that for human growth hormone-growth hormone receptor (figure 1.4). Site-directed mutagenesis data from cytokine receptors (review by Young 1992) imply a third potential receptor-binding site in the LEP-R. From the crystalline structure of leptin-E100 this third position has been proposed to be on a site containing the BC and CD loops (marked on the top of the helix D in figure 1.4).

1.7 Leptin secretion and metabolism

Leptin is secreted from the adipose tissue into the circulation, where it is measured using immunoprecipitation assay or radioimmunoassay (Maffei *et al.* 1995; Considine *et al.* 1996b; Ma *et al.* 1996; Hosoda *et al.* 1996). Licinio *et al.* (1997) suggest that the secretion of leptin is pulsatile and has a circadian rhythm, with the highest levels occuring at night. Serum leptin circulates, in part, bound to transport proteins (Houseknecht *et al.* 1996; Sinha *et al.* 1996) and the proportion of free leptin in the circulation increases with increasing obesity (Houseknecht *et al.* 1996).
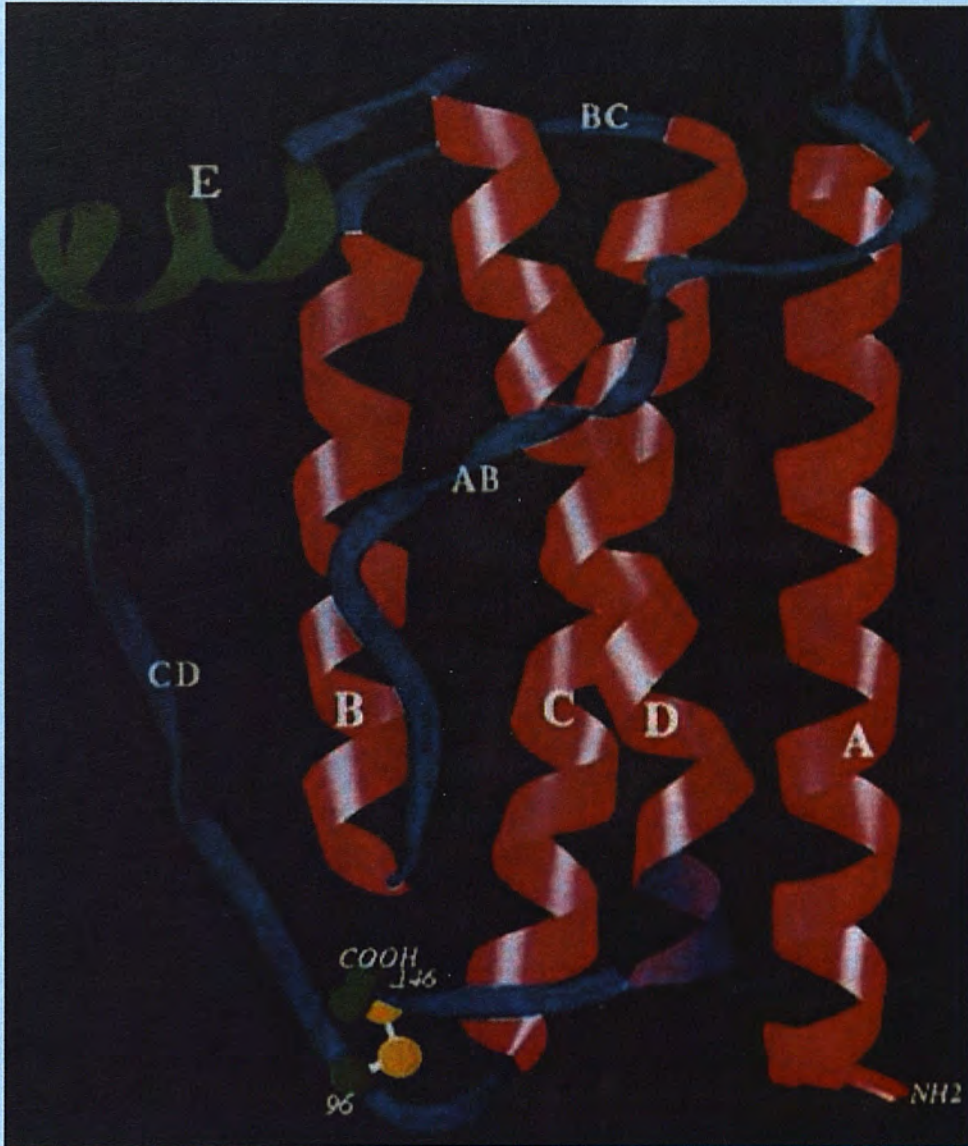
Figure1.3 Helical structure of leptin. The helical structure of leptin
deduced from a mutant form, with a substitution of Glu for Trp at position
100 known as leptin E100. The view is perpendicular to the four-helix
bundle axis. The four α-helices (A,B,C, and D) are shown in red; the bent
C terminal 310 helical extension of helix D is highlighted in magenta; the
additional short helical segment E is green. The connecting loops AB, BC,
and CD are coloured cyan. The termini are marked with NH2 and COOH,
respectively. The disulfide bridge between the C-terminal Cys 146 and Cys
96 in the AB loop is represented with a ball-and-stick model. (From: Zhang
et al. 1997).

Figure 1.4. Leptin and Leptin Receptor Binding- Model. Model of receptor binding sites of leptin protein (cyan ribbon) superimposed on the position of Growth Hormone (GH) (magenta ribbon) in the GH/GHR complex. The two receptor binding sites are marked as site1 and site 2 along the residues of the GH receptor. A third putative receptor binding site is shown by the arrow near the BC and CD loops on the top of the helix D. The orientation is rotated 90° from Figure 3 (From: Zhang *et al.* 1997).

Many factors (age, sex, body composition, pubertal status, and nutritional status) affect serum leptin concentrations. The concentration of serum leptin shows sexual dimorphism, with women having higher levels than men, independent of body adiposity (Havel *et al.* 1996; Rossenbaum *et al.* 1996). This may be due to a higher ratio of subcutaneous to omental fat mass in women than in men (Bouchard *et al.* 1993), and to sex differences in reproductive steroid hormones (Havel *et al.* 1996). This view is supported by studies which suggest a negative association between serum leptin and testosterone levels (Jockenhovel *et al.* 1997; Behre *et al.* 1997).

The concentration of serum leptin is highly correlated with adipose tissue mass. In humans and mice, serum leptin decreases after weight loss (Maffei *et al.* 1995) and increases as the size of the adipose tissue triglyceride stores increases (Maffei *et al.* 1995; Considine *et al.* 1996b). In rodents, an increase in caloric intake results in an acute increase in serum leptin, approximately 40% over base line within 12h in the absence of a change in body weight (Sinha *et al.* 1996; Kolaczynzki *et al.* 1996a, b). In contrast, serum leptin levels in humans do not increase acutely in the postprandial state (Kolaczynski *et al.* 1996b), although they decrease in response to starvation (Kolaczynski *et al.* 1996a; Boden *et al.* 1996). Concentrations of serum leptin are high in obese humans and in several genetic and environmentally induced forms of obesity in rodents (Maffei *et al.* 1995). Leptin has a half-life in the serum of approximately 90 minutes.

1.8 Leptin receptor

Studies of leptin gene expression in mice with brain lesions led to the conclusion that leptin's effects on appetite are mediated via the hypothalamus, a brain region known to control body weight (Maffei *et al.* 1995). Following the discovery that I^{125}-labelled leptin binds specifically to the choroid plexus, a murine choroid plexus cDNA library was constructed, and cells transfected with this library were screened with a leptin-alkaline phosphatase fusion protein. From this screening, cDNAs were identified that encoded a cell surface leptin receptor (LEP-R), classified as a cytokine receptor (Tartaglia *et al.*

1995). The leptin receptor gene maps to the same region of mouse chromosome 4 that contains the *db* locus (Tartaglia *et al.* 1995).

The structure of the leptin receptor is similar to that of the class I cytokine receptor family, which includes receptors for growth hormone (GH), prolactin (PRL), interleukin-6 (IL6), the leukaemia inhibitory factor (LIF), ciliary neurotropic factor (CNTF). Leptin receptor shows a close resemblance to gp130, the common signal-transducing subunit of a subgroup of cytokine receptors (figure 1.5). The first member of the leptin receptor family to be identified was LEP-Ra (figure 1.5), which has a single transmembrane domain (Tartaglia 1995). Further screening and analysis of cDNA libraries using the LEP-Ra cDNA sequence resulted in the discovery of multiple forms of LEPR in mice and humans (figure 1.5). The murine leptin receptor gene encodes at least seven splice variants: LEP-Ra, LEP-Rb, LEP-Rc, LEP-Rd, LEP-Re, LEP-Rf* (figure 1.5) and *muB219*. A long form (LEP-Rb or LEP-R$_L$) has a signal-transducing intracellular motif of 303 amino acids, which the other splice variants lack (Chen *et al.* 1996; Lee *et al.* 1996; Cioffi *et al.* 1996). This intracellular domain of the long form contains sequence motifs suggestive of intracellular signal-transducing capabilities (Tartaglia 1997).

The gene encoding long form of the leptin receptor, LEP-Rb, is expressed at a high level in the arcuate, dorsomedial, ventromedial and paraventricular nuclei of hypothalamus (Mercer *et al.* 1996; Lee *et al.* 1996) and at a lower levels in hind brain, including the nucleus of the solitary tract, lateral parabrachial nucleus and medullary reticular nucleus. All these nuclei are implicated in processing satiety and energetic signals of peripheral origin (Mercer *et al.* 1998). The long isoform of the leptin receptor is, therefore, thought to mediate the central actions of leptin in the regulation of food intake. The long isoform of leptin receptor is also localized in anterior pituitary gland (Cai and Hyde 1998), in ovarian granulosa and thecal cells (Karlsson *et al.* 1997) and in the inner zone of the kidney and the adrenal medulla (Hoggard *et al.* 1997a).

All the isoforms of leptin receptor, identified to date, have identical extracellular ligand binding domains (figure 1.5) (Tartaglia *et al.* 1995; Lee *et al.* 1996). The functions of the
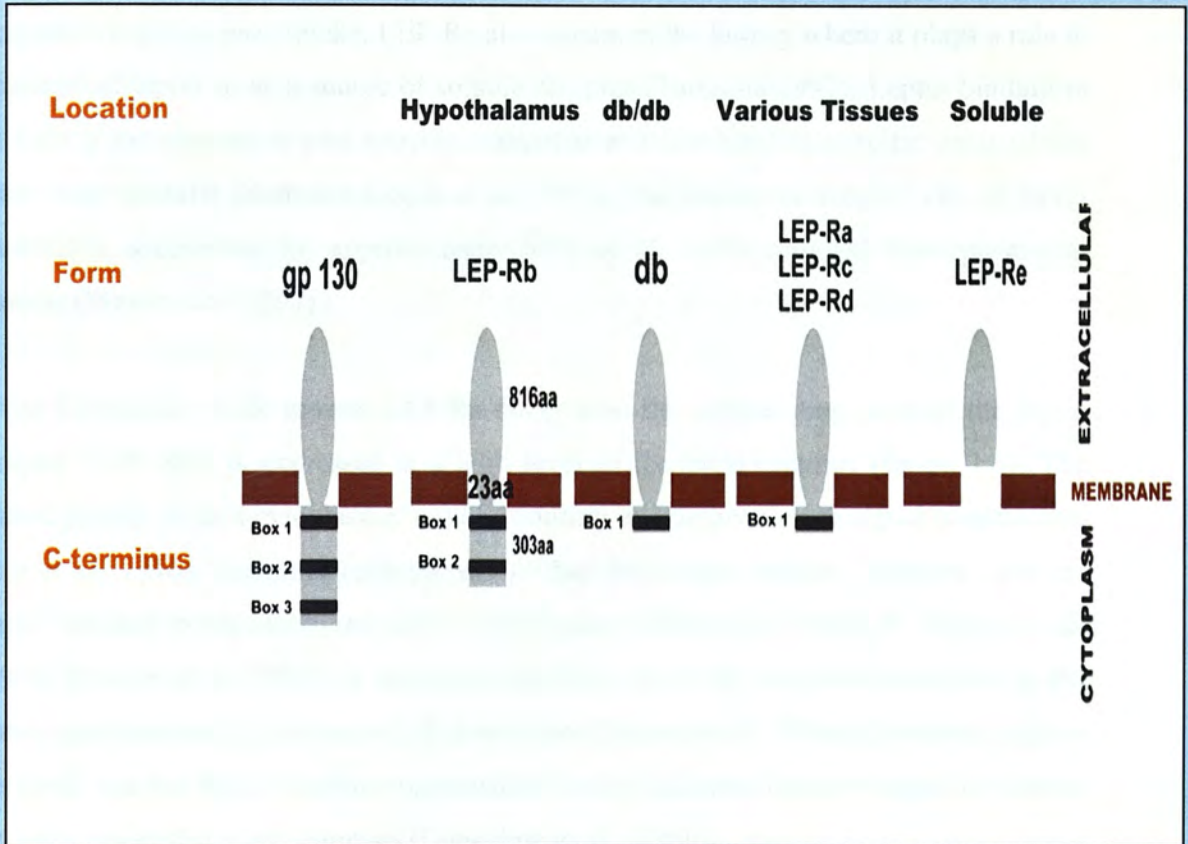
Figure 1.5 Schematic representation of leptin receptor isoforms and the structurally related gp130 receptor. Different forms of the leptin receptor that arise by alternative splicing of leptin receptor mRNA are denoted as LEP-Ra, LEP-Rb, LEP-Rc, LEP-Rd and LEP-Re. All share identical extracellular , ligand-binding domains but they differ at the C terminus. Only LEP-Rb, the long isoform, encodes all protein motifs capable of activating the JAK -STAT signal pathway. All but LEP-Re, the soluble form, have the transmembrane domain. LEP-Re is secreted as a truncated form. In C57B1/Ks db/db mice, the mutation of the LEP-R gene causes a premature stop codon in the LEP-Rb 3' end, resulting in the replacement of the LEP-Rb form with LEP-Ra. Box 1-3 indicate amino acid sequence motifs suggestive of intracellular signal-transducing capabilities (Data from Tartaglia 1997).

forms including LEP-Ra with short intracellular domains remains to be defined. Since leptin enters the brain by a specific and saturable transport mechanism (Banks *et al.* 1996; Golden *et al.* 1997) it is likely that the high levels of LEP-Ra in the choroid plexus plays a role in transporting leptin from the blood to the cerebrospinal fluid (CSF). Once in the CSF, leptin may then directly access brain areas containing the long form of leptin receptor to regulate food intake. LEP-Ra also occurs in the kidney where it plays a role in clearance of leptin or as a source of soluble receptor (Tartaglia 1997). Leptin binding to rat kidney membranes is also specific, saturable and localized to specific areas of the inner renal medulla (Serradeil-LeGal *et al.* 1997). The kidney is a major site of leptin catabolism, accounting for approximately 80% of all leptin removal from plasma in humans (Meyer *et al.* 1997).

In the C57BL/Ks db/db mouse, LEP-Ra rather than the normal long form of the leptin receptor (LEP-Rb), is expressed at a high level in the hypothalamus (figure 1.5). The mutant protein lacks a cytoplasmic region resulting in defective leptin signal transduction (Lee et al. 1996). Genomic analysis shows that the mutant mouse "diabetes" and rat "fatty" are due to the mutations of the LEP-R gene (Chua *et al.* 1996a, b; Takaya *et al.* 1996b; Phillips *et al.* 1996). A nonsense mutation of LEP-R has been identified in the obese, spontaneously hypertensive, Koletsky rat (Takaya *et al.* 1996a). However, neither the db/db nor the fa/fa mutations responsible for the defective leptin receptor in rodents has been reported in obese humans (Considine *et al.* 1996b).

1.9 Leptin signal transduction pathway

The homology between leptin and class I cytokine receptors suggests the identity of the intracellular mediators of leptin receptor activation. Class I cytokine receptors typically lack intrinsic tyrosine kinase activity and are activated by the formation of homo- or heterodimers (Watowich *et al.* 1996). These receptors act through signal transducers and activators of transcription (STAT) and Janus kinase (JAK) proteins (Kishimoto *et al.* 1994). Intracellular signalling is mediated by cytoplasmic JAK proteins, which are

autophoshorylated in response to ligand-receptor binding. The activated JAK proteins then phosphorylate specific tyrosine residues on the receptor's cytoplasmic tail, providing binding sites for STAT proteins (Ihle 1995).

There is substantial evidence that the leptin signal transduction pathway involves STAT and JAK proteins. In cells transfected with LEP-Rb, treatment with leptin activates STAT 3 and 5 (Bauman *et al.* 1996; Ghilardi *et al.* 1996). Leptin also induces a dose-dependent activation of the transcription factor STAT-3 in the hypothalamus of mice within 15 minutes of a single intraperitoneal injection (Vaisse *et al.* 1996). Further, tyrosine phosphorylation of STAT 1 is induced in a human renal carcinoma cell line after treatment with leptin (Takahashi *et al.* 1996). Leptin also increases the expression of fos, a STAT-3 target (Woods and Stock 1996).

In addition to STAT/JAK signal transduction, other leptin signalling pathways are known. Synaptic transmission in the arcuate nucleus is influenced by leptin and glucose (Glaum *et al.* 1996) leading to the hyperpolarization of some hypothalamic neurons. This effect depends on an ATP-dependent potassium channel, since tolbutamide, which blocks these channels, inhibits this effect (Spanswick *et al.* 1997). These electrophysiological effects of leptin are rapid and unlikely to involve activation of STAT proteins (Friedman and Halaas 1998).

It might be assumed that the long but not the short form of LEP-R is capable of leptin signal transduction. This is not the case. The ubiquitous presence of the truncated leptin receptor in almost all tissues, and the direct effects of leptin on several peripheral tissues (Lee *et al.* 1996; Lollman *et al.* 1997; Bjorbaek *et al.* 1997) suggest that the short isoforms (LEP-Rs) may also mediate actions of leptin. Several studies are consistent with this view. When expressed in CHO cells, the short isoform LEP-Ra mediates leptin-induced expression of the early genes c-fos, c-jun and jun-B (Murakami *et al.* 1997) where both LEP-R forms mediate leptin-induced phosphorylation of JAK2 (Bjorbaek *et al.* 1997). Leptin also stimulates tyrosine phosphorylation of insulin receptor substrate-1

through both the long and short forms of the leptin receptor (Bjorbaek *et al.* 1997). Thus, both long and short forms of leptin receptor mediate leptin signal transduction. Although the short forms have an attenuated signal, their presence in a variety of peripheral tissues suggests that they mediate specific peripheral effects of leptin.

Some of leptin's actions in the brain may be mediated by interleukin-1 and prostaglandins, since the suppressive effect of leptin on fever, but not food intake, is abolished by a cyclooxygenase inhibitor, which inhibits prostaglandin synthesis (Luheshi *et al.* 1999). Thus, in addition to its role in regulating body weight, leptin may mediate neuroimmune responses via actions in the brain, which depend on the release of interleukin-1 and prostaglandins.

1.10 Leptin resistance

Resistance to the biological action of leptin is caused by several mechanisms. First, the discovery that mutations in the leptin and leptin receptor genes cause severe obesity in rodents suggests that similar mutations could be one cause of obesity in man. However, with the exception of *ob/ob* mouse and two children (Montague *et al.* 1997), which have mutated leptin genes, most examples of rodent and human obesity are characterized by hyperleptinaemia (reviewed by Caro *et al.* 1996; Spiegelman and Flier 1996). This suggests that mutations in the leptin receptor gene and/or in genes associated with signal transduction pathway, rather than in the leptin gene, are more likely to be the genetic cause of leptin resistance. The diabetes (*db/db*) mouse is the typical example, where a defect in leptin receptor expression or proximal signalling events in the brain results in leptin resistance.

Secondly, leptin resistance may be due to change in the way leptin is transported in the blood. Many members of the cytokine family circulate bound to proteins in serum. These binding proteins may regulate hormone clearance rates, increasing or decreasing biological activity of the ligand, and/or provide hormone responsiveness to otherwise unresponsive cells (Heaney and Golde 1993; Bonner and Brody 1995). Thus, the capacity

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of serum binding proteins to transport leptin may be a critical feature of leptin resistance. In support of this view the difference in serum leptin levels between obese and lean individuals is greater than in the cerebrospinal fluid (Schwartz *et al.* 1996; Caro *et al.* 1996).

Thirdly, leptin resistance may simply reflect the limitations of the leptin "system" to regulate food intake and body fat stores (Houseknecht *et al.* 1998).

1.11 Physiological actions of leptin

1.11.1 Central actions

Leptin derives its name from its ability to induce weight loss and provides a hormonal signal from adipose tissue (figure 1.6) to the brain to coordinate the neuroendocrine response to starvation (Ahima et al. 1996). Administration of leptin to *ob/ob* mice producing truncated, non-functional leptin, results in sharp decrease in weight, mediated through reduced food intake, increased energy expenditure and increased thermogenesis (Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995). Normal lean mice also lose weight in response to leptin administration, but the magnitude of this response is not as dramatic as that in *ob/ob* mice (Pelleymounter *et al.* 1995). Intra-cerebroventricular administration of leptin results in a more potent response than that seen after systemic administration, consistent with the view that the CNS is a major site of action of leptin (Schwartz *et al.* 1996a). Further evidence for the central action of leptin comes from the observation that systemic leptin administration in rats induces the expression of c-fos, a putative marker for neuronal activation, in hypothalamic and brain stem areas known to be involved in the regulation of appetite (Elmquist *et al.* 1997).

Among leptin-sensitive cells in the hypothalamus are arcuate nucleus neurons containing neuropeptide Y (NPY), agouti-related peptide (AGRP), and proopiomelanocortin
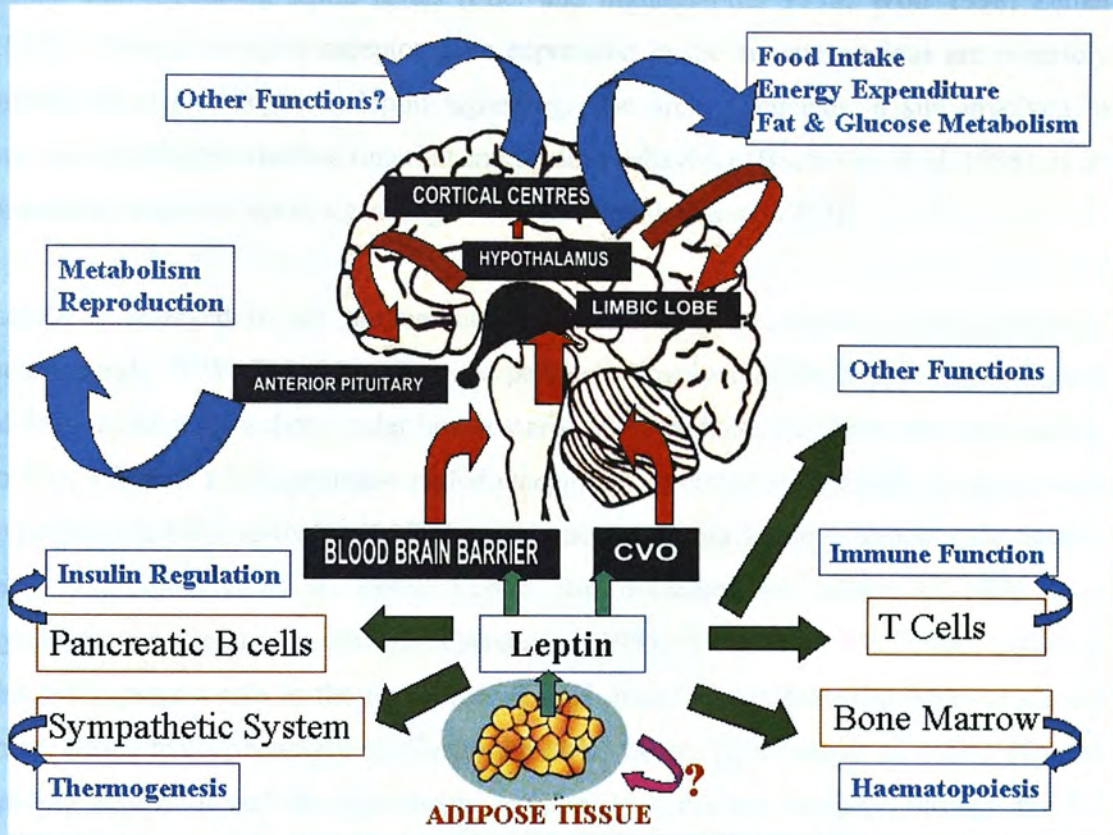
Figure 1.6 Physiological Functions of Leptin. Leptin is the afferent signal in a negative feedback loop that maintain constant body fat. It is secreted from adipocytes either as a 16K protein or bound to a soluble form of its receptors. Leptin enters the brain chiefly through the blood brain barrier and also via the circum ventricular organ (CVO). It acts mainly on the hypothalamus which has extensive projections to other brain regions. Leptin acts centrally to reduce food intake and modulates glucose and fat metabolism. Through the anterior pituitary it regulates reproductive functions. Peripheral actions include regulation of insulin secretion and nutritional function, thermogenesis through brown adipose tissue and sympathetic system, immune functions in nutritional stress, haematopoiesis, vasculogenesis, direct actions in the gut and gonadal functions (Taken from Friedman and Halaas 1998; Hossner 1998).

(POMC), the precursor of the catabolic peptide, alpha melanocyte stimulating hormone (αMSH) (Baskin *et al.* 1999). Cells producing these peptides contain leptin receptor mRNA and show changes in neuropeptide gene expression in response to changes in food intake and circulating leptin levels (Flier and Maratos-Flier 1998; Wolf 1998; Zemel 1998). Changes in leptin receptor gene expression in the arcuate nucleus are inversely associated with changes in leptin signalling. The arcuate nucleus, a site involved in control of both reproductive function and feeding behaviour (Buchanan *et al.* 1998), is an important target for leptin signalling in the brain (Baskin *et al.* 1999).

Leptin is believed to act predominantly by inhibiting the action of the orexigenic neuropeptide, NPY. This 36-amino-acid peptide is involved in the hypothalamic control of food intake and cardiovascular homeostasis. NPY mediates its effects through binding to Y1-, Y2- and Y5- G-protein-coupled receptors (Naveilhan *et al.* 1999). In *ob/ob* mice hypothalamic NPY mRNA and NPY peptide decrease after leptin treatment (Stephans *et al.* 1995; Schwartz *et al.* 1996). Leptin also decreases the release of NPY from hypothalamus explants in vitro (Stephens *et al.* 1995; Wang *et al.* 1997). Key evidence that NPY plays a role in the regulation of food intake comes from the NPY-knock out mice, which has a phenotype similar to the *ob/ob* mouse (Erickson *et al.* 1996). Normal feeding behaviour and the hypophagic response to leptin are mediated through the Y2 receptor (Naveilhan *et al.* 1999). Thus, a change in NPY gene expression appears to play a role in mediating the actions of leptin in the CNS. However, food intake in NPY-knock out mice is depressed after leptin administration to the same extent as it is in wild type mice. This observation suggests that NPY is not the only neuromodulator mediating the actions of leptin in the CNS (Erickson *et al.* 1996).

Other hypothalamic factors known to mediate the central actions of leptin include melanocyte stimulating hormone (MSH), its receptor, melanocortin-4 (MC-4), and agouti-related protein (AGRP) (Friedman and Halaas 1998). Abnormal melanocortin signaling in yellow agouti (A^Y) or MC-4-knockout mice is characterized by obesity and leptin resistance (Halaas *et al.* 1997; Fan *et al.* 1997; Huszar *et al.* 1997). A subset of hypothalamic neurons express both LEP-R and proopiomelanocortin (POMC), the

precursor of MSH, and this co-localization explains how leptin modulates POMC gene expression (Hakansson *et al.* 1998). Agonists of MC-4 (e.g. αMSH) decrease food intake while α-MSH antagonists (e.g. AGRP) blunt the anorexic effect of leptin (Satoh *et al.* 1998).

Evidence that AGRP is implicated in the regulation of body weight is supported by the observation that transgenic mice overexpressing AGRP are markedly obese (Ollman *et al.* 1997). Further, levels of mRNA encoding AGRP are increased eightfold in *ob/ob* mice (Shutter *et al.* 1997). A single i.c.v. injection of AGRP in rats significantly increases cumulative food intake and body weight in a dose-dependent manner (Ebihara *et al.* 1999). Leptin-induced inhibition of food intake and body weight gain is reversed by co-injection of AGRP in a dose-dependent manner (Ebihara *et al.* 1999). Leptin modulates POMC gene expression to increase hypothalamic α-MSH production (Hakansson *et al.* 1998), suggesting that the action of leptin via the hypothalamic melanocortin system is determined by the balance between the levels of its agonist, α-MSH and antagonist, AGRP.

Another hypothalamic hormone involved in the central action of leptin is the corticotropin-releasing factor (CRF). CRF is the primary hypothalamic hormone stimulating the release of pituitary adrenocorticotropic hormone (ACTH) which in turn regulates glucocorticoid secretion from the adrenal glands. Neurons and terminals containing CRF, and high-affinity binding sites for CRF, are localized in various regions of the brain (Clegg and Smagin 1999). Leptin increases levels of corticotropin-releasing factor (CRF) mRNA in the hypothalamic paraventricular nucleus (PVN) and stimulates the release of CRF from perfused brain slices containing the amygdala or the PVN (Schwartz *et al.* 1996; Elmquist *et al.* 1997). Delivery of CRF to the PVN results in reduced food intake and increased energy expenditure in lean and obese rats. Pretreatment with anti-CRF antibodies decreases the anorexic effect of a single i.c.v. dose of leptin (Gardner *et al.* 1998).

Urocortin, a recently described member of the CRF family has 45% sequence similarity with CRF and has been shown to be more potent than CRF in suppressing feeding (Clegg and Smagin 1999). Leptin administration increases the expression of specific CRF receptors. It is likely that leptin, NPY and CRF/urocortin interact to control satiety and regulate body weight. High levels of serum glucocorticoids are seen in most strains of genetically obese mice, and adrenalectomy and glucocorticoid antagonists reduce obesity in *ob/ob, db/db,* and other obese mice (Freedman *et al.* 1989). Replacement therapy with low doses of glucocorticoid restores obesity indicating that glucocorticoids have a permissive role in the development of the obese phenotype. It is not known whether the requirement for glucocorticoids in the development of the full *ob/ob* phenotype is a consequence of a suppression of CRF release or of some other effect of glucocorticoids.

Insulin (Woods *et al.* 1996), and the hypothalamic neuropeptides, bombesin (Ohki-Hamazaki *et al.* 1997), melanin-concentrating hormone (MCH) (Qu *et al.* 1996), cocaine-and amphetamine-regulated transcript (CART) (Kristensen *et al.* 1998), orexin-A and orexin-B (Mondal *et al.* 1999) are all thought to play a role in the regulation of appetite (Spiegelman and Flier 1996; Hirschberg 1998; Sahu 1998; Xu *et al.* 1998). Leptin may interact with all these regulatory factors. Cholecystokinin (CCK), considered as a physiological 'satiety peptide' from the gut and brain, potentiates the anorexic effect of leptin (Matson *et al.* 1997). In addition, exogenously administered sulfated octapeptide of CCK (CCK-8) decreases stores of leptin in the stomach wall (Bado *et al.* 1998).

In summary, concentrations of serum leptin are sensed by groups of neurons, which are characterized by multiple neuropeptides in the hypothalamus. Starvation decreases serum leptin, which activates behavioral, hormonal and metabolic responses that are adaptive when food is in short supply. When food is plentiful and body weight increases, the concentration of serum leptin increases to elicit a different hypothalamic response, resulting in a state of negative energy balance. It is not yet known whether the same (or different) neurons respond to increasing and decreasing leptin levels. The range of leptin's effects is likely to be complex, since different thresholds are known to exist for several of leptin's actions (Ioffe *et al.* 1998).

1.11.2 Peripheral action

Leptin directly affects the functions of adipose and other peripheral tissues. Leptin receptor isoforms are expressed in adipose tissue, bone marrow, pancreatic tissue, and vascular epithelia (Tartaglia *et al.* 1995; Lee *et al.* 1996; Chen *et al.* 1996; Karlsson *et al.* 1997; Hoggard *et al.* 1997a; Ghilardi *et al.* 1996). Leptin reduces lipid synthesis in cultured adipocytes and decreases triglyceride synthesis and increases fatty acid oxidation in cultured pancreatic islet cells (Bai *et al.* 1996; Shimabukuro *et al.* 1997). Biological responses to leptin are observed in cultured hepatocytes (Cohen *et al.* 1996b), adipocytes (Bai *et al.* 1996), haemopoietic cells (Ghilardi and Skoda 1997; Gainsfold and Alexander 1999), and pancreatic islet cells (Shimabukuro *et al.* 1997). Additionally, leptin modulates insulin secretion as well as insulin-regulated responses suggesting a negative feedback loop between leptin and insulin (Keifer *et al.* 1997). In hepatocytes, leptin decreases insulin receptor substrate-1 phosphorylation, modulates downstream effectors of insulin action and upregulates gluconeogenesis (Cohen *et al.* 1996b). About 70% of the postprandial increase in insulin secretion is mediated by peptide hormones such as glucagon-like peptide-1 (GLP-1) derived from the gut. In the gut, leptin inhibits GLP-1 secretion and in pancreatic β-cells it reduces the insulinotropic effect of GLP-1 (Fehman and Goke 1997), and thus leptin causes severe postprandial hyperglycaemia in non-insulin-dependent diabetes mellitus.

It is usually assumed that leptin controls body fat by decreasing food intake and increasing energy expenditure, but a few studies demonstrate that leptin increases the energy expenditure of lean adult animals (Schmidt *et al.* 1997; Pelleymounter *et al.* 1995; Stehling *et al.* 1996). For example, although leptin does not influence metabolic rate or body temperature in wild type mice, a stimulatory effect on the metabolic rate and body temperature is seen in *ob/ob* mice (Pelleymounter *et al.* 1995). Further, leptin treatment reduces the body fat in suckling-age rat pups by an amount corresponding to the increase in energy expenditure (Stehling *et al.* 1996). The amplitude of the daily rhythm of metabolic rate is smaller in rat pups treated with leptin than in control pups, although the

food intakes of control and leptin-treated pups are identical (Stehling *et al.* 1996). An effect of leptin on metabolic heat production in rat pups becomes obvious (Stehling *et al.* 1997) when thermoregulatory activation is suddenly elicited by briefly subjecting them to cold conditions. In contrast, in adult free-feeding mice maintained under moderately cold conditions, leptin administration does not increase the metabolic rate to a level higher than that normally attained under moderate cold conditions (Pelleymounter *et al.* 1995). In summary, leptin attenuates temporary depressions in cold-induced, sympathetically mediated thermogenesis, such as those occurring during the early morning hours in food-restricted adult mice (Hudson 1978; Himms-Hagen 1985), or in rat pups reared by their mother or artificially and maintained at ambient temperatures below thermoneutrality (Redlin *et al.* 1992; Nuesslein-Hildesheim and Schmidt 1994).

Leptin (Wang *et al.* 1998) and leptin receptor (Hoggard *et al.* 1997) gene expression occurs in skeletal muscle in mammals. In isolated mouse soleus muscle, leptin stimulates fatty acid oxidation by 42% while reducing fatty acid incorporation into triglycerides by 35% (Muoio *et al.* 1997). The mouse embryonic cell line CEH10T1/2, which can differentiate into muscle, fat or cartilage, is induced to proliferate by leptin via the mitogen-activated protein kinase system (Takahashi *et al.* 1997). Also, leptin upregulates uncoupling protein-3 (UCP-3) expression in skeletal muscle (Liu *et al.* 1998). These studies provide evidence of a direct effect of leptin on skeletal muscle and show that leptin not only regulates lipid and carbohydrate metabolism, but also plays a fundamental role in modulating cell proliferation, embryonic development and energy metabolism (Hossner 1998).

The leptin receptors present in bone marrow may play a role in immune responsiveness, haematopoiesis, and osteogenesis (Pighetti *et al.* 1999). Leptin stimulates haematopoesis *in vitro* by inducing proliferation, differentiation and functional activation of hemopoietic cells (Gainsford *et al.* 1996). The vascular endothelium is also a target for leptin since leptin induces angiogenesis (Sierra-Honigmann *et al.* 1998). Leptin may have both hypertensive action by enhancing sympathetic activity and antihypertensive action by

promoting renal excretion of sodium and water (Haynes *et al.* 1998). Leptin receptors in bone growth plates play a role in osteogenesis (Pighetti 1999).

1.11.3 Evidence from gene therapy studies for the physiological functions of leptin

Gene therapy has been used to assess the physiological effects of inducing chronic hyperleptinaemia in both leptin-deficient and normal animals. In one such experiment, *ob/ob* mice were treated with a recombinant adenovirus expressing the mouse leptin cDNA (Muzzin *et al.* 1996). The treatment resulted in dramatic reductions in both food intake and body weight, as well as in normalization of serum insulin levels and glucose tolerance. After the treatment finished, the subsequent decrease in serum leptin levels resulted in the rapid increase in food intake and gradual gain in body weight, which correlated with the progressive return of hyperinsulinaemia and insulin resistence. In another study, hyperleptinaemia was induced in normal Wistar rats for 28 days by infusing a recombinant adenovirus expressing rat leptin cDNA (Chen *et al.* 1996). The hyperleptinaemic rats reduced in food intake by 30-50% and gained 22 g body weight during the experimental period. Control animals, which were given saline infusions or a control recombinant virus expressing the β-galactosidase gene, gained 115-132 g body weight during the same period.

1.12 Leptin in reproduction and development

The classical studies of Kennedy and Mitra and the work of Frisch and her co-workers established that the timing of sexual maturation is associated with a critical body weight and composition (Kennedy and Mitra 1963; Frisch *et al.* 1973). Since the brain receives and processes metabolic cues, and sexual maturation is initiated only when body energy reserves are adequate to meet the demands of mating, pregnancy and lactation (Wade and Schneider 1992), leptin is a primary candidate for the metabolic cue required to stimulate reproductive function. Evidence for this role of leptin comes from the observation that leptin treatment rescues the infertile phenotype of *ob/ob* mice (Pelleymounter *et al.* 1995;

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Barash et al. 1996; Chehab et al. 1996). Further, leptin treatment of mice accelerates the onset of puberty (Ahima et al. 1997), possibly by decreasing the inhibitory feedback of oestrogen acting upon gonadotropin secretion (Barash et al. 1996; Chehab et al. 1997). Leptin is not seen as the primary signal that initiates the onset of puberty, but rather as a permissive factor allowing pubertal maturation to proceed (Barash et al. 1996; Cheung et al. 1997).

Leptin acts on several components of the reproductive system including the hypothalamic-pituitary-gonadal axis, the uterus and placenta (figure 1.7). It also acts in the foetus to promote growth and metabolism (figure 1.7). An action of leptin at neuroendocrine level of the reproductive system is suggested by the demonstration that leptin (116-130), an active fragment of the native molecule, stimulates LH and PRL secretion in fasted adult male rats (Gonzalez et al. 1999). An action of leptin directly in the ovary is suggested by the finding that leptin receptor mRNA occurs in the ovary (Cioffi et al. 1996). Functional leptin receptors including the long isoform are expressed in ovarian granulosa and thecal cells (Karlsson et al. 1997). Further, leptin impairs the insulin-like growth factor-I (IGF-I)-mediated augmentation of follicle stimulating hormone (FSH)-stimulated oestradiol-17β synthesis by the granulosa cells (Zachow and Magoffin 1997). Leptin also acts on thecal cells to increase insulin-induced proliferation and block insulin-induced progesterone and androstenedione production (Spicer and Francisco 1998). The inhibitory effect of leptin on steroidogenesis appears to be mediated through binding to its receptor.

In addition to being secreted from adipose tissue, leptin is also produced in other tissues such as the placenta (Masuzaki et al. 1997; Ashworth et al. 2000). Leptin produced by the placenta has the same size and immunoreactivity as leptin produced by adipose tissue (Bodner et al. 1999), whereas its expression appears to be regulated by a placenta-specific enhancer (Bi et al. 1997). Placental leptin is angiogenic and immunomodulatory and plays an important role in regulating maternal energy balance during late pregnancy and in maternal-fetal interactions during intrauterine development (Hassink et al. 1997; Holness et al. 1999; Himmshagen 1999; Ashworth et al. 2000).
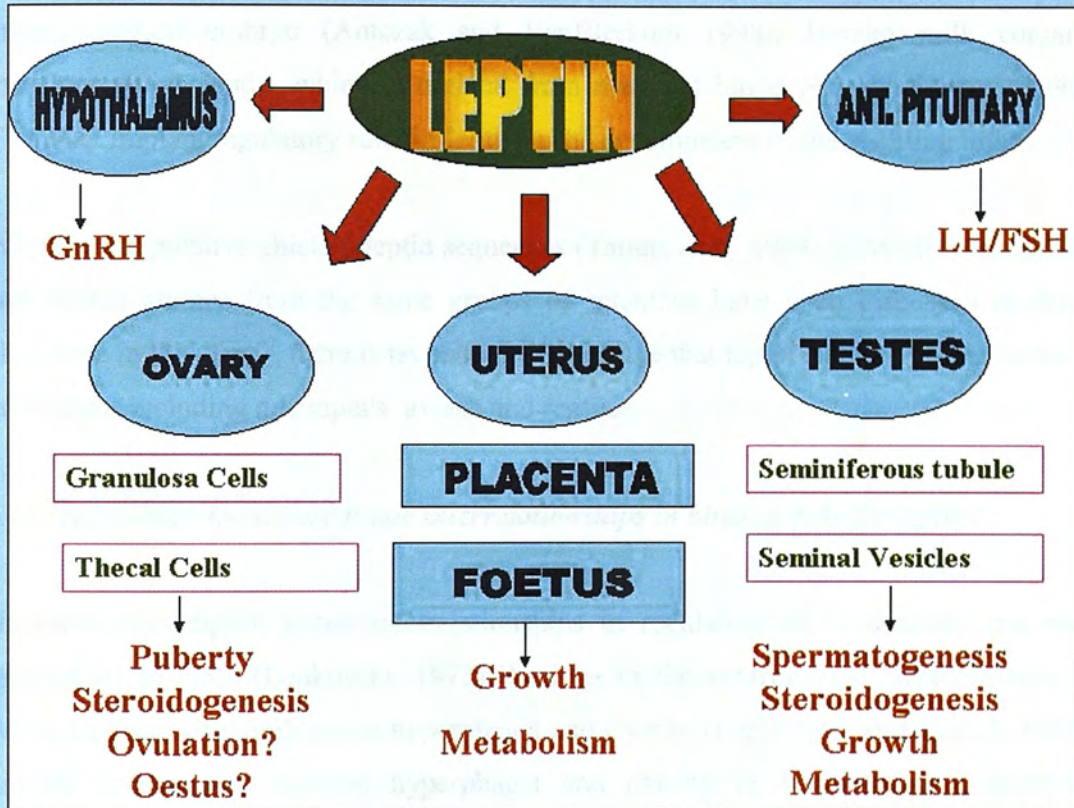
Figure 1.7 Effects of leptin on reproductive and developmental process.

Leptin is synthesised in the human foetus particularly in cartilage/bone and hair follicles (Hoggard *et al.* 1997) and foetal serum leptin correlates with foetal body weight gain (Harigya *et al.* 1997). The level of leptin in the umbilical cord blood correlates directly with birth weight (Schubring *et al.* 1997). Leptin and STAT3 have critical roles in early mammalian development, and may be involved in the determination of the animal pole of the oocyte, and establishment of the inner cell mass and trophoblast in the preimplantation embryo (Antczak and VanBlerkom 1998). Human milk contains immunoreactive leptin, which is derived from maternal blood (Casabiell *et al.* 1997). This may imply a regulatory role for leptin in the development of the suckling infant.

Whereas the putative chicken leptin sequences (Taouis *et al.* 1998; Ashwell *et al.* 1999a) and related studies from the same groups of scientists have been discussed in detail elsewhere in this thesis, there is no published evidence that leptin exists in lower forms of vertebrates including marsupials, avians and reptiles.

1.13 Hypothalamic-adipose tissue interrelationships in birds: a role for leptin?

Hypothalamic-adipose tissue interrelationships in regulation of food intake are well established in birds (Lepkovsky 1973). Lesions in the ventromedial hypothalamus of White Leghorn cockerels cause hyperphagia and obesity (Lepkovsky and Yasuda 1966). Similar lesions also induced hyperphagia and obesity in White-throated sparrows (*Zonotrichia albicolis*) (Kuenzel and Helms 1970). Hypophagia and weight loss occurs in chickens (Smith 1969) and White-throated sparrows (Kuenzel 1972) with lesions in the lateral hypothalamus.

The metabolic signal acting upon the chicken hypothalamus to control feed intake is likely to be a blood-borne factor. In support of this view, intracerebroventricular (i.c.v.) injections of concentrated plasma from domestic chicken fed *ad libitum* reduces the food intake of normal birds whereas plasma from 24h-fasted birds does not affect food intake (Skewes et al. 1984). Further, a low molecular weight (< 1500) serum fraction from Leghorn cockerels fed ad libitum has a satiating effect in domestic chicken (Skewes *et al.*

1986). The discovery of leptin as a major satiety signal in mammals (Zhang *et al.* 1994) suggests that in birds, an avian form of this hormone may serve a similar function. A zooblot observation by Zhang *et al.* provides compelling evidence that a leptin-like gene sequence occurs in the chicken genome (figure 1.8).

1.14 The potential commercial significance of leptin in poultry

If leptin affects body fat composition, feed intake, muscle growth, development, and immune responsiveness in chicken, as in mammals, the identification of genes encoding chicken leptin and its receptors will be of commercial importance. Carcass composition, rate of growth, feed conversion ratios and reproductive potential are key genetic traits of interest to poultry producers.

Chickens reared to produce eggs for human consumption eat to maintain body weight and egg production. Feed conversion efficiency is a key commercial trait in these birds. Feed conversion efficiency is also important in broiler chickens bred for meat production, but these birds feed in an unregulated manner resulting not only in rapid growth but in excessive fat deposition. Female broiler breeder birds fed *ad libitum* deposit so much internal fat that normal egg production is impaired (Nahm and Chung 1995). Excessive fatness results in leg deformities and impairment of testicular function in adult broiler males and impairment of ovarian function in adult females which decrease the fertility of breeding flocks, particularly after 50 weeks of age (Etches 1996).

In order to improve the fertility of broiler breeders, the birds are subjected to restricted feeding to produce pullets that are not excessively fat when they lay their first egg or start producing semen (Nahm and Chung 1995). The large size and enormous appetite of broiler breeders, especially the males, necessitates complex management systems that must take into account sexual dimorphism and body size, hunger and sexual and aggressive behaviour (Etches 1996). An understanding of the physiology of leptin in the broiler could lead to better management systems for maximum fertility. Further, genes encoding leptin and its receptor(s) are potential candidates within quantitative trait locus
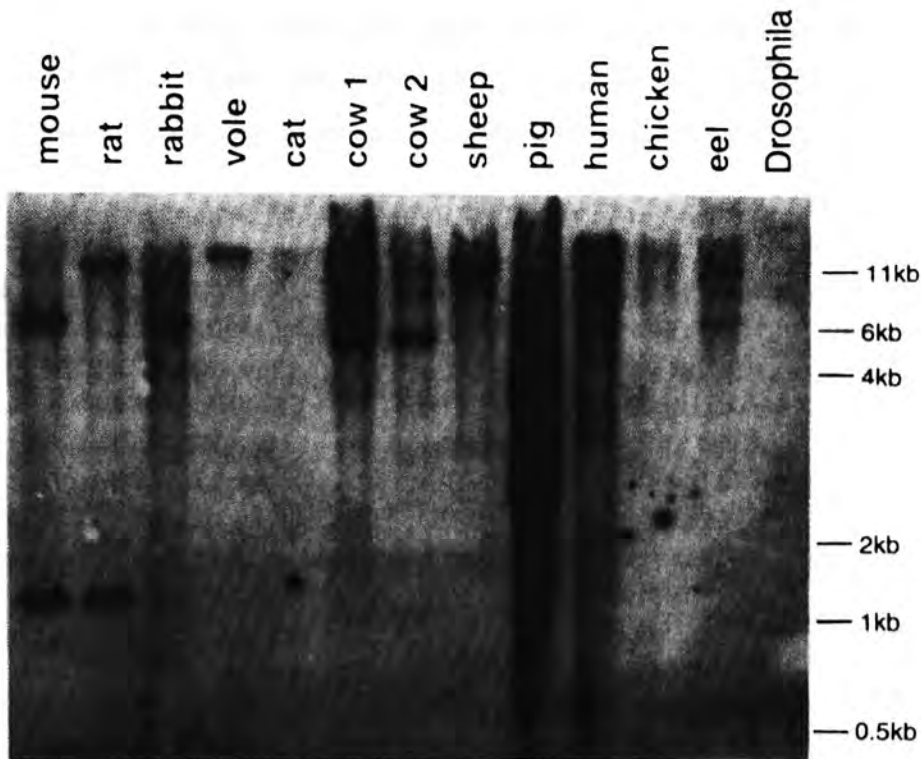
Figure 1.8: Zooblot presented by the authors reporting the positional cloning of leptin gene in mouse and man. Cross-species hybridization of genomic DNA blots with mouse leptin probe indicated evolutionary conservation of leptin gene. Note the band indicating hybridization to a leptin-like sequence in the chicken genome (Ref: Zhang *et al.* 1994).

(QTL) currently being identified at Roslin Institute for growth rate, feed conversion efficiency, body composition and reproductive performance.

A gene encoding chicken leptin was reported during the course of this work (Taouis *et al.* 1998; Ashwell *et al.* 1999). The chicken leptin-like cDNA sequence reported is 95% identical at the nucleotide with mouse leptin cDNA. As discussed in chapter 7 our understanding of evolutionary molecular biology is such that it is highly improbable that there is such a high homology between mouse and chicken leptin genes.

1.15 Research objective

The present study was undertaken to establish the existence of a leptin gene homologue in the domestic chicken (*Gallus gallus*) genome, and to determine the degree of homology of the sequence with the mammalian leptin genes and with published chicken leptin sequences.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and standard solutions

2.1.1 Laboratory Chemicals

General laboratory chemicals were obtained from Fisons (Scotlab, Coatbridge, Strathclyde, U.K.), BDH or Sigma (Poole, Dorset, U.K.), unless indicated. All solutions were made up in MQ purified water (see below).

2.1.2 MQ water

This was particle-free (Δ 0.22 μm), reagent grade (<10ppb TOC, ISO3696/BS3978- Grade 1) water purified at 18.2 megohm-cm resistivity by Milli-Q RG water purification system, Millipore Limited, Watford, Hertfordshire, UK.

2.1.3 Standard solutions

<u>10% Ammonium persulfate</u>: Ammonium persulfate in water (1 g/10 ml) and stored at 4°C.

<u>Ampicillin</u>: 1 g/10 ml in water used as stock.

<u>BBL-Bottom agar</u>: Trypticase 10 g (Becton Dickinson, Cowley, Oxford, U.K.), agar 10 g (Difco Labs, East Molesey, Surrey), and NaCl 10 g, per litre of water.

<u>BBL-Top agar</u>: Trypticase 10 g, agar 7 g (Difco Labs, East Molesey, Surrey), and NaCl 10 g, per 1 litre water.

<u>Denhardt's solution</u>: Ficoll 0.02%, polyvinylpyrolidone 0.02%, bovine serum albumin 0.02%, in water.

0.5M EDTA: Disodium ethylene diamine tetra acetate.$2H_2O$ 186.1 g in 880 ml of water, adjusted the pH to 8.0 with NaOH pellets and then the volume adjusted to 1 litre using water. Aliquots (100 ml) were sterilized by autoclaving before storage at 4°C.

Equilibrated phenol: Redistilled phenol equilibrated with equal volumes of 0.5 M Tris (pH 7.5) until the pH was greater than 7.0. Stored with an equal volume of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and 0.1 % hydroxyquinoline at -20°C.

Ethidium bromide: Ethidium bromide 1 g/100 ml in water dissolved over stirrer for several hours to ensure that the dye had dissolved. Stored in a bottle wrapped in aluminium foil and stored at room temperature. Since ethidium bromide is toxic, gloves and a mask were worn when preparing this solution.

Gel loading dye: Glycerol 50%, 2% Tris acetate electrophoresis buffer 50%, bromophenol blue 2.5 mg, xylene cyanol 2.5 mg in 10 ml.

Isopropylthio-β-D-galactoside (IPTG): IPTG 2 g dissolved in MQ water (10 ml). The solution was sterilized by passing through a 0.22 μm disposable filter.

L-Agar: Agar 15 g, tryptone 10 g, yeast extract 5 g (Difco Labs, East Molesey, Surrey), NaCl 5 g in a litre of water. The pH was adjusted to 7.2 with NaOH.

L-Broth: Tryptone 10 g, yeast extract 5 g (Difco Labs, East Molesey, Surrey), NaCl 5 g in a litre of water. The pH was adjusted to 7.2 with NaOH.

20% Maltose solution: Maltose 20 g in water 100 ml. The solution was sterilized by filtering through a 0.22 μm filter and stored at room temperature.

NZY Bottom agar: NaCl 5 g, $MgSO_4.7H_2O$ 2 g (Fisons Analytical Grade Reagent), NZ amine or casein hydrolysate 10 g (Sigma), yeast Extract 5 g, and agar 5 g (Difco Labs, East Molesey, Surrey), in a litre of water. The pH was adjusted to 7.5 with NaOH.

NZY Top agar: NaCl 5 g, $MgSO_4.7H_2O$ 2 g (Fisons Analytical Grade Reagent), NZ amine or casein hydrolysate 10 g (Sigma), yeast extract 5 g, and agar 5.6 g (Difco Labs, East Molesey, Surrey), in a litre of water. The pH was adjusted to 7.5 with NaOH.

Phage buffer: 22 mM KH_2PO_4, 50 mM Na_2HPO_4, 85 mM NaCl, 1mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001% gelatin (Sigma).

SM buffer: NaCl 5.8 g, $MgSO_4.7H_2O$ 2 g, 1 M Tris.HCl 50 ml (pH 7.5) and 2% (w/v) gelatine 5 ml (Sigma) in a litre of water.

3M Sodium acetate (pH 5.2): Sodium acetate.3H$_2$O 408.1 g, water 800 ml. The pH was adjusted to 5.2 with glacial acetic acid and made up to 1 litre with water. The solution was dispensed into aliquots of 100 ml and sterilized by autoclaving.

10% Sodium dodecyl sulfate (SDS): Electrophoresis-grade SDS 100 g, water 900 ml. This was placed in a microwave oven to solubilize. The pH was adjusted to 7.2 with HCl and then made up to 1 litre with water. The solution was dispensed into aliquots of 100 ml and stored at room temperature.

SOB Medium: Tryptone 20 g, yeast extract 5 g (Baltimore Biologicals) and NaCl 0.5 g in water 950 ml. The mixture was shaken until clear. 250 mM KCl (10 ml) was added and the volume was made up to one litre with water. The solution was sterilized by autoclaving.

SOC Medium: SOB medium was autoclaved and cooled to 60°C or less, then a 0.22 μm filter-sterile glucose solution was added to a concentration of 20mM.

SSC: A solution containing 15 mM Na$_3$ citrate and 150 mM NaCl in water.

Tris EDTA (TE): A solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA in water.

Tris acetate electrophoresis buffer (TAE): A solution containing 40 mMTris-acetate and 1 mM EDTA in water.

Tris borate electrophoresis buffer (TBE): A solution containing 89 mM Tris-borate and 2.5 mM EDTA in water.

X-gal (5-Bromo-4chloro-3-inodyl-β-D-galactoside): X-gal dissolved in dimethyl formamide 20 mg/ml. The solution was stored in polypropylene tubes wrapped in aluminium foil and stored at -20°C.

2.2 Animals and Tissues

2.2.1 Laboratory animals

Adult male and female C57BL6 mice were obtained from Roslin Institute's breeding stock; male and female broiler (Cobb and Ross lines), bantams, White Leghorn chickens and male and female Japanese quail were obtained from stocks maintained at Roslin Institute. Scottish black face sheep were from the Roslin Institute's flock. All animals were fed *ad libitum* and under a * 14L/10D lighting schedule.

2.2.2 Tissues from laboratory animals

Avian blood was collected with EDTA as the anticoagulant. Soft tissues were snap-frozen in liquid nitrogen and kept frozen at -70°C until used for RNA and DNA extraction.

2.3 E.coli Host Strains and Vectors

2.3.1 E.coli strain

The strains of *E.coli* were BB4, DH5-α and XL1-Blue, all from Stratagene (Cambridge, UK).

2.3.2 Plasmid

The plasmid was Bluescript SKII⁺ (pBSKII⁺) (Stratagene Ltd, Cambridge, UK.)

2.4 Restriction Enzymes and Molecular Weight Markers

2.4.1 Restriction enzymes

Restriction enzymes were supplied by Boehringer Mannheim GmbH (Biochemica, Mannheim, Germany) Northumberland Biologicals (Morpeth, Northumberland, U.K.) or Pharmacia (Milton Keynes, Bucks, U.K.) unless otherwise mentioned. The enzymes were stored frozen at -20°C and handled with rubber gloves. The enzymes were dispensed using pipettes with filter tips. These precautions were taken to avoid DNA contamination.

2.4.2 Molecular weight markers

2.4.2.1 Low Molecular weight Markers

Reagents for a low molecular weight DNA marker ladder (100 bp Ladder plus) were purchased from MBI Fermentas, Vilnius, Lithuania, through Helena Biosciences Ltd., UK. According to the manufacturer's specifications the restriction digested, phenol extracted and ethanol precipitated plasmid DNA was dissolved in 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA to give a concentration of 0.3-0.5 mg DNA/ml. The resulting DNA marker ladder contained 14 bands of 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200 and 100. The amount of DNA fragments in the 500bp band was always the highest resulting in the most prominent band in the DNA ladder. Thus the 500 bp band was used as a reference for identifying other marker bands.

2.4.2.2 High molecular weight marker

Reagents for a high molecular weight DNA marker ladder, Lamba DNA/Bsp68I (NruI)/XhoI Marker12, were purchased from MBI Fermentas, Vilnius, Lithuania through Helena Biosciences Ltd., UK. According to the manufacturer's specifications the restriction digested, phenol extracted and ethanol-precipitated plasmid DNA was dissolved in 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA to give a concentration of 0.3-0.5 mgDNA/ml. The Bsp 68I and XhoI digest of DNA yielded eight discrete bands at 33498, 23460, 15004, 9401, 6694, 4590, 3653 and 704 base pairs.

2.2.2.3 Gel loading dye

The 6× loading dye consisted of bromphenol blue 0.25%, xylene cyanol 0.25%, glycerol 30%, and EDTA 50 mM.

2.5 Radionucleotides

2.5.1 Radionucleotides

Redivue [α^{32}P]-labelled dCTP (220TBq/mmol, 37.0 MBq/100 µl), [α ^{35}S] dATP and α ^{35}S-labelled dATP (110TBq/mmol, 37.0 MBq/100 µl) were purchased from Amersham

International plc, Buckinghamshire, UK. They were stored in 4°C and exposure of the reagents to room temperature was kept to a minimum.

2.5.2 Radioactive contamination

After use, all the hybridization chambers were rinsed in a sink before monitoring with a Geiger counter. If a chamber was still radioactive (showing > 50 in the Geiger counter) it was wiped down with a tissue soaked in Decon 90 and checked again for radioactive contamination. If a chamber was still radioactive after this treatment, it was soaked in 5% solution of Decon 90 for a few hours and rinsed in water again.

2.6 Genomic DNA Library

2.6.1 Chicken genomic cosmid library

A gridded chicken cosmid library prepared from white and red blood cells from a laying Rhode Island Red chicken using superCos 1 as the vector and DH5α strain of E.coli as the host, was a gift from Dr. Leonard Cornelis Schalkwyk, Prof. Dr. Hans Rudolf Fries, Dr. Michael Weiher and Johannes Buitkamp (Tech University, Munich, Lehrstuhl Tierzucht, Freising, Germany and Max Planck Institute of Molecular Genetics, Berlin, Germany). This library provides a powerful tool for rapid physical mapping and complex analysis of the chicken genome (Buitkamp *et al.* 1998). About 110,000 cosmid clones were grown and replicated in 384-well plates. An analysis of 68 randomly selected clones showed that the average insert size was 39kb and the range of the insert size was 20-48 kb. It is calculated that the library provides a four-fold coverage of the genome.

2.7 Extraction of genomic DNA(gDNA)

Mouse genomic DNA (gDNA) was prepared using a commercial kit (Nucleon ST kit, Amersham Life Science) according to the manufacturer's instruction.

Sheep genomic DNA from embryonic liver was kindly provided by Dr. L. Young, Roslin Institute.

Chicken gDNA was prepared from whole blood. Samples were collected in syringes rinsed with 0.5 M Na_2EDTA and transferred into tubes containing 0.5 M Na_2EDTA (5 μl). These samples were either immediately processed or stored at 4°C. Each blood sample (100 μl) diluted in 300 μl reagent A (10 mM Tris/HCl , pH 8.0; 0.32 M sucrose; 5 mM $MgCl_2$; 1% triton X-100; adjusted to pH 8.0 with NaOH and autoclaved) in Eppendorf tubes and mixed for 5 min at room temperature. The tube was centrifuged for 3 min at 2,500 rpm on a microfuge at room temperature. The supernatant was discarded taking care not to disturb the loose pellet. A 1 ml aliquot of reagent B (400 mM Tris/HCl pH8.0; 60 mM Na_2 EDTA; 150 mM NaCl; 1% Sodium dodecylsulfate) was added to the tube and the pellet was gently resuspended by pipetting in and out using a wide bore tip. The tube was incubated at 37°C for 60 min after adding 5 μl of RNAseA solution (final concentration 50 μg/ml). Sodium perchlorate (5 M, 250 μl) was then added and the tube was shaken at room temperature for 15 min. Next, the tube was transferred to a 65°C water bath and incubated for a further 25 min with frequent shaking. Chloroform (300 μl) (previously stored at -20°C) was added and the tube was shaken gently for 5 min. After centrifugation at 13000 rpm for 5 min, the upper aqueous phase (800 μl) was removed to a labelled tube, taking care not to disturb the interphase layer. Isopropanol (640 μl) was added and the tube was shaken gently until the DNA precipitated. The DNA was pelletted by microfugation at 13,000 rpm for 5 min. The supernatant was discarded and the pellet washed in 70% ethanol before drying under vacuum for 15 min.

To ensure complete solubilization, water was added to the extracted DNA (approximately 1 ml/mg) and was maintained in a shaken incubator at 37°C overnight. The solubilized DNA samples were stored frozen at -20°C. The optical density at 260 and 280 nm was used to calculate the 260/280 ratio and the yield of DNA. The quality of the genomic DNA was assessed by subjecting the sample to overnight electrophoresis on a 0.7% normal agarose gel in 1× TAE containing ethidium bromide at 25-30 volts.

The gDNA samples were stored at -70°C.

2.8 Extraction of RNA

After dissection, tissues were snap-frozen in liquid nitrogen, before storage at -70°C. RNA extraction was performed using RNAzolB (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. The samples were homogenized manually in 500 μl RNAzolB using a disposable pellet pestle (Anachem Ltd, Bedfordshire, UK.). A further 500 μl RNAzolB together with 150 μl of chloroform were added. The tubes were vortexed and left on ice for 15 min before being centrifuged at 4°C for 15 min at 13,000 rpm. Seven hundred microlitres of the aqueous phase were transferred to a fresh tube and mixed at room temperature with 700 μl isopropyl alcohol. After 45 min incubation at -20°C, the tubes were centrifuged for 15 min at 13,000 rpm at 4°C. The pellet was washed with 50% isopropyl alcohol, followed by 70% ethanol and dried in a vacuum desiccator. After dissolving in water, the optical density at 260 and 280 nm was determined and an aliquot of the total RNA was run on a 1.4% agarose gel. The RNA samples were stored at -70°C.

2.9 Agarose Gel Electrophoresis

2.9.1 Double stranded DNA

Separation of DNA by agarose gel electrophoresis was performed in submarine electrophoresis tanks (BRL, Paisley, Strathclyde, UK) using 1%TAE running buffer. Agarose gels between 0.8 and 3% were used or, if preparative gels were required, a similar percentage of low melting point agarose were used. In addition to these two types of agarose, 3 or 4% NuSieve agarose gels (Flowgen Instruments Ltd, Sittingbourne, Kent, UK.) were used. NuSieve gels, designed to separate small fragments of DNA between 20 -2,000 bp, have a low melting point and are free of the impurities in standard agarose, which inhibit enzyme activity.

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These gels were used for direct 'in-gel' cloning and kinasing of small DNA fragments without further purification. In all cases samples were mixed with gel loading dye (20-50%) before running.

2.9.2 RNA

RNA electrophoresis was performed using the non-formaldehyde method of Pelle and Murphy (1993). The RNA samples (1 to 10 µg), dissolved in water (10 µl), were mixed with RNAse-free loading buffer (2 µl) [6× loading buffer = 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30%(w/v) glycerol, 1.2% SDS, 60 mM sodium phosphate (pH6.8)], and incubated at 75ºC for 5min. Samples were then immediately loaded onto a 1.4% agarose gel containing 0.1 µg/ml ethidium bromide. When analysing many samples, the denatured RNA was placed on ice before loading. The gel was electrophoresed in 10 mM sodium phosphate buffer, pH 6.8, containing ethidium bromide (0.1 µg/µl) at 3 to 7 V/cm. Because the buffering capacity of the electrophoretic buffer is weak due to its low ionic strength, the buffer was recirculated continuously to prevent the formation of a pH gradient, which could lead to degradation of the RNA during electrophoresis. The RNA was visualised under UV illumination.

2.10 Recovery and Purification of DNA

DNA fragments amplified by PCR were recovered and purified from solutions or agarose gels using a GeneClean kit (Bio101, Anachem Ltd, Luton, Beds, UK) according to the manufacturer's instructions. The procedure was performed in a 1.5 ml Eppendorf tube. For agarose gel samples, a volume of sodium iodide (NaI) corresponding to three times the weight of the agarose was added, and the tube was incubated for 5-10 min at 45-55ºC until the agarose dissolved. For DNA in solution, the volume of NaI was three times that of the sample solution, and it was not necessary to incubate the tube. After addition of NaI, glass milk suspension (5 µl) (both supplied in the kit) was added to the tubes and they were then

incubated for 5 min at room temperature with frequent vortexing. Tubes were microfuged at 13,000 rpm for 5sec. in order to pellet the glassmilk/DNA complex. "NEW WASH" solution (750 µl) (supplied in the kit) was then added to the tubes, before microfuging. Each time the glassmilk was resuspended by rinsing with a pipette. This process was repeated thrice, with the glassmilk pellet being resuspended by rinsing with a pipette. The remaining "NEW WASH" solution was then removed with a pipette and the tubes were dried under vacuum for 10 min before being eluted into water.

2.11 Extraction of Miniprep

Bacterial colonies were picked with a toothpick and transferred into a 1.5 ml Eppendorf tube containing 1 ml L-broth plus ampicillin (140 µl of the stock/100ml broth). After overnight culture at 37°C, tubes were centrifuged for 1min to pellet the cells. The supernatant was gently decanted, leaving 50-100 ml of L-Broth together with the cell pellet. The tube was vortexed at high speed for 5-10 sec to resuspend the cells completely. Three hundred microlitres of TENS solution (10 mM Tris, 1 mM Na_2 EDTA, 0.1 M NaOH, 0.5% sodiumdodecyl sulphate) was then added and the tube was vortexed for 2-5 sec until the mixture became sticky. Sodium acetate (150 µl, 3 M, pH 5.2) was added to the tube on ice before vortexing for 2-5 sec. The tube was then microfuged for 4 min at 13,000 rpm to pellet cell debris and chromosomal DNA. The supernatant was transferred to a marked tube and mixed well with absolute ethanol (900 µl), which had been pre-cooled to -20°C. Plasmid DNA and RNA were pelleted by spinning the tube for 6min at 13,000 rpm. The supernatant was discarded, the pellet rinsed once with 70% ethanol (1ml), and then dried under vacuum until all traces of ethanol were removed (5-10 min). The plasmid DNA and RNA pellet were resuspended in 30-50 µl of RNAse A solution in water (100 mg/ml). The tube was further incubated in a 37°C water bath for 30 min. The plasmid DNA solution was stored frozen at -20°C.

2.12 Restriction enzyme digestion

Restriction enzymes were obtained from Boehringer Manneheim (BCL, Lewes, Sussex,UK) or New England Biolabs (New England Biolabs/C.P Laboratories, Bishop's Stortford, Hertfordshire, UK) unless mentioned otherwise. Digestions were carried out as recommended by the manufacturers using the buffers supplied.

2.13 Ligation reactions

Ligations were carried out overnight at 37°C in a volume of 10 μl containing 1% ligation buffer (66 mM Tris [pH 7.2], 1 mM EDTA, 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP) and one unit of T4 DNA ligase (New England Biolabs, CP Labs. Bishop Stortford, Herts, U.K.). The amount of insert used in ligations depended on the nature of the free ends; typically in a sticky ended ligation a two fold excess of insert to vector was used. 'In gel' ligation was carried out overnight at room temperature with DNA fragments separated on NuSieve gels (Flowgen Inst. Ltd, Sittingbourne, Kent, U.K.). The gel was melted and diluted 3-fold with MQ water before mixing at 37°C with the other ligation solutions.

2.14 Transformation of E.coli (Hanahan Method)

A 1/50 dilution of an *E.coli* overnight culture was used as a starter to produce 50 ml of a log phase culture after incubation in SOB (50ml) (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 7.0) which was previously sterilized by autoclaving in suitable sized aliquots. Just before use, 1 M $MgCl_2$ was added to produce a concentration of 20 mM Mg^{2+}. The SOB inoculated with *E.coli* was incubated for approximately 3 h at 37°C until the OD_{650} = 0.5. The culture was further incubated for at least 15 min at 4°C after which the cells were separated by centrifugation (2,800 rpm) at 4°C and the cell pellet was resuspended in 100 mM

TFB (16 ml) (10 mM MES, 100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM hexamine cobalt chloride) and left on ice for 15 min. The cells were centrifuged again at 4°C, and resuspended in TFB (4 ml). A 140 µl aliquot of DnD (1 M dithiothreitol; DMSO 90% (v/v); 0.01 M potassium acetate pH7.5, made up in water) was then added and the cells were incubated for 15 min at 4°C. A further 140 µl of DnD was added and the incubation continued for a further 15 min. before the cells were ready for use. Up to 5ng of DNA was mixed with 200 µl of the competent cells and incubated for at least 30 min at 4°C. The cells were then heat-shocked for 90sec at 42°C and returned to ice for at least 15 min. If the transformed cells were to be subjected to ampicillin resistance selection they were incubated at 37°C for 45 min with 1ml of SOC (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM Mg SO_4, 20 mM glucose) to allow the expression of the ampicillin resistance gene before plating out on a selective medium.

2.15 Transfer of DNA to Nylon Membranes

2.15.1 Prebloting preparation of the agarose gel

For depurination, the gel block was rinsed in water before being immersed in 10× the gel volume of 0.25 M HCl for 30min The gel was rinsed again in water before transfer to 10× the gel volume of 0.4 M NaOH for 20min for complete denaturation of the genomic DNA. Finally, the gel was washed in water before proceeding with the blotting.

2.15.2 Blotting by downward capillary transfer

Capillary transfer of the gDNA using alkaline buffer was achieved by both upward (Sambrook et al. 1989) and downward (Ausubel et al. 1998) transfer, but the downward method described was more effective. A stack of paper towels, larger in dimension than the gel, 2-3cm high, was made on a Saran wrap polythene sheet spread on a plastic tray. Four pieces of Whatman 3 MM filter paper (larger than the gel dimensions but slightly smaller than the paper towels) were placed on top of the paper towels. A fifth filter paper was moistened in the transfer buffer (0.4

M NaOH) and placed on top of the Whatman filters. An Amersham Hybond N$^+$ (positively charged) nylon membrane (other membranes tested, Amersham Hybond N and Hybond XL, were found to be less effective) was cut to a size just larger than the gel, and one corner was removed and marked to orientate the membrane after blotting. The membrane was moistened with water and placed on top of the wet filter paper. Bubbles were removed by rolling a glass tube over the surface of the membrane. Four small strips of polythene wrap were placed over the edges of the membrane to prevent short-circuiting. The corner of the gel was cut to correspond to the cut corner on the membrane and was then placed over the membrane with the polythene wrap on the edges, taking care that no part of the gel extended beyond the edges of the membrane. Three pieces of Whatman 3 MM filter paper (cut to the exact size as the gel) were moistened in the transfer medium (0.4 M NaOH) and placed on top of the gel. Two longer pieces of Whatman 3 MM filter paper both soaked in transfer buffer were placed on top of the others to act as a wick, bridging the top of the gel to a dish containing a reservoir of transfer buffer. An electrophoresis dish was placed on top of the wet filters, to prevent evaporation, and left for 60-90 min to complete the transfer of DNA into the nylon membrane.

2.15.3 Immobilization of DNA

After blotting, the membrane was rinsed in 2 × SSC, placed on a sheet of freshly cut Whatman 3MM filter paper and allowed to air dry as specified by the manufacturers. The membrane was stored wrapped in 3MM paper in a dry place at room temperature.

2.16 Preparation of DNA Probes by Random Priming

Random primed labeling, based on the method of Feinberg and Vogelstein (1983, 1984) was employed to incorporate radioactive nucleotides along the length of a fragment of template cDNA to be used as the probe.

2.16.1 Random primed ^{32}P-labelling of DNA

The following reagents and materials were used to prepare random primed ^{32}P-labelled DNA:-

1. The mouse leptin probe template was a 348 base-pair mouse leptin cDNA probe extending from positions 134 to 481 of the mouse leptin mRNA sequence (Zhang *et al.* 1994). This was generated by RT-PCR of mouse fat RNA with primers RF2 and RR1 (see section *2.17.3*).

2. The chicken glyceraldehyde 3-phoshate dehydrogenase (GAPDH) cDNA probe template (344bp between nucleotide 900 and 1243 of the chicken GAPDH mRNA, accession number-KO1458, Version: KO1458.1 GI:211800 of Genbank) was prepared from the plasmid construct with the chicken GAPDH sequence insert by PCR amplification using the following primers: Forward: GAPDHFor (19mer)- TGT GAC TTC AAT GGT GAC A. Reverse: GAPDHRev (19mer)- GCT GAT AGA AAC TGA TCT G.

[The PCR amplified template DNA was run on a 3% agarose gel and the bands were cut out from the gel and purified using the Geneclean kit (Anachem Ltd., Vista, CA). Template concentration was determined by absorbance at 260 nm on a spectrophotometer and the sequence was determined using a Thermosequenase cycle sequencing kit (Amersham Life Science, UK).]

3. The oligo labelling buffer (OLB) was made up from:- Solution O: 1.25 MTris-HCl, pH 8.0, 0.125 M $MgCl_2$ (stored at 40°C). Solution A: 2β-mercaptoethanol (18 µl), dATP (5 µl), dTTP (5 µl) and dGTP (5 µl) were added to 1 ml of solution O. Solution B: 2 M HEPES, titrated to pH 6.6 with 4 M NaOH (store at 4°C). Solution C: hexadeoxyribonucleotides evenly suspended at 90 $0D_{260}$ 50 U/ml (stored at -20°C). Solutions A, B, and C were mixed in a ratio of 100:250:150 and stored at -20°C.

4. Redivue [α ^{32}P] dCTP- 220TBq/mmol, 37.0 MBq/100 µl was purchased from Amersham Pharmacia, Amersham International plc, Buckinghamshire, UK and stored at 4°C.

5. The Klenow fragment of DNA polymerase1 (5 U/µl) was purchased from New England Biolabs, Hertfordshire, UK, and stored at -20°C.

6. Bovine serum albumin (Sigma, Poole, Dorset, UK) was prepared at a concentration of 10 mg/ml in water.

2.16.1.2 Method 1

Approximately 30 ng of the template DNA, which was to be labelled (the probe) was placed in water (33 µl) in a 1.5 ml Eppendorf tube. The DNA was denatured by placing the tube in a waterbath at 95°C for 3 min, and then into crushed ice for 2min. The tube was then microfuged to bring the contents to the bottom. To the denatured and diluted DNA template solution from the previous step, were added the following: OLB buffer (10µl), 10 mg/ml BSA (1 µl), labelled nucleotide (5 µl), and Klenow fragment (1 µl). All the components were mixed together by gentle pipetting in and out. The tube was then incubated at room temperature for 4-5 h.

2.16.1.3 Method 2

The DNA template solution (7.5 ng DNA/10 µl) was heated at 95°C for 3 min and then kept on crushed ice for 2 min. The tube was centrifuged to bring the contents to the bottom. Denatured template DNA solution (30 µl) was added to a 1.5 ml Eppendorf tube containing 5% OLB Buffer (10 µl), purified BSA (1 µl), Klenow fragment (1 µl) and $[\alpha\ ^{32}P]$ dCTP (5 µl). The volume of the tube was made up to 50 µl using water. After vortexing, the tube was incubated at room temperature in a perspex box for more than 3 h.

2.16.2 Phenol-chloroform extraction

Labelled probe DNA was added to an Eppendorf tube, followed by equilibrated phenol (30 µl, kept at 4°C) and 30 µl of chloroform (30 µl, kept at -20°C) and then vortexed. The contents of the tube were spun to the bottom by microfugation. The tube was placed in a dry heating block at 70°C for 2 min, vortexed again and replaced in the heated block for another 5 min. The tube was finally vortexed and microfuged at 13.000 rpm for 4 min.

2.16.3 Spinning through a Sephadex column

After chloroform-phenol extraction, the supernatant aqueous phase (50 µl) was pipetted onto the top of a TE Midi Select-D G-50 Sephadex column (5 Prime 3 Prime Inc., CP Labs, Bishops Stortford, Herts, UK) placed in a collection tube. After 2 min at room temperature, the collection tube and column was microfuged at 13,000 rpm for 1min. The column was then removed from the collection tube, leaving the probe solution.

2.16.4 Evaluation of the probe

2.16.4.1 Evaluating radioactivity

The probe solution was diluted 1/200 by mixing 0.5µl with 99.5 µl of water. The diluted probe solution (10 µl) was mixed with 3ml of a scintillation cocktail fluid (Fisher Chemicals, Loughborough Leicester, UK) and counted on a beta counter (Wallac-1410 ,Wallac Oy, Turku 10, Finland). The cocktail fluid was counted as a control.

2.16.4.2 Electrophoresis and autoradiography

The probe solution (1-2 µl) was loaded on a 3% agarose mini gel with a low molecular weight marker, and run for less than 1 h. Thereafter, the gel was exposed to UV light to view the probe. The gel was then vacuum-dried on a stack of Whatman 3MM blotting papers and wrapped in saran wrap. Once dried, the gel was loaded against a cleaned phosphor imager screen for 2 h. The phosphor imager screen was developed to confirm the size (position) and pattern of the signal generated by the probe.

2.17 Polymerase Chain Reaction

2.17.1 General conditions, chemicals and equipments

PCR were performed on a Hybaid OmniGene thermal cycler (Hybaid Ltd, Middlesex, UK). Reagents and Taq DNA polymerase were from GibcoBRL (Life Technologies Ltd, Fountain Drive, Paisley, UK) and Boehringer Mannheim GmbH (Biochemica, Mannheim, Germany). A typical PCR was performed in 25 μl of a solution containing 1× magnesium-free PCR buffer, 1-3 mM magnesium, 200μM of each dNTP, and 1 μM of each primer together with 1 unit of Taq polymerase. When touch-down conditions were used, the annealing temperature was reduced by 0.5°C in successive one-cycle steps from 64°C to 60°C, or from 60°C to 56°C. Denaturing and extension temperatures were 94°C and 72°C respectively. Times used were 15 s for denaturation, 30 s for annealing and 45 s for extension. After a temperature of 60°C or 56°C was reached, a further 30 cycles were carried out under the same conditions. The extension time for the final cycle was 5 min. PCR products were visualized by agarose gel electrophoresis and further characterised by Southern hybridization with mouse leptin probes, or by subcloning and sequencing. Amplified fragments were ligated into pBluescript II SK$^+$ plasmid (Stratagene Ltd, Cambridge, UK.), transformed into *E.coli* XL1-Blue (Stratagene Ltd, Cambridge , UK.), and sequenced with a Thermo Sequenase cycle-sequencing kit (Amersham Pharmacia Biotech, Amersham International plc, Buckinghamshire, UK).

2.17.2 PCR templates

Reverse-transcribed RNA or genomic DNA samples were used as templates for amplification by PCR. Genomic templates were typically used at a concentration of 20 ng 125 μl reaction. Immediately prior to reverse-transcription, all RNA samples were incubated with 4 U Dnase I (Roche Diagnostics, Lewes, East Sussex, UK) at 37°C for 30 min to remove any residual genomic DNA, followed by heat-inactivation of the DNAseI at 75°C for 10 min.

2.17.3 PCR primers

Sixteen primers were prepared and used in different combinations. The position of these primers relative to the mouse leptin sequence is shown in figure 3.2. These were complementary to the 5' and 3' ends of the sequence to be amplified and were purchased from Cruachem, Glasgow, Strathclyde, UK. Lyophilized primers were reconstituted in water to a

concentration of 100 pmol/μl according to the manufacturer's instructions. Reconstituted primer samples were divided into small aliquots and stored at -20°C.

An anchored oligo(dT) T18 NN adaptor primer was also used.

2.18 Reverse transcription PCR

Reverse transcription polymerase chain reaction (RT-PCR) (Frohman *et al.* 1988; 1989) uses the same protocols as standard PCR except that the target DNA is derived from RNA. Total RNA was prepared using the RNAzolB kit (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instruction. The RNA samples were heated at 65°C for 10 min and put on ice immediately to cool. The total RNA sample, containing up to 2.5μg of RNA in 4 μl was placed in a 0.5 ml microcentrifuge tube. Reverse transcription was performed using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Amersham International plc, Buckinghamshire, UK.). One microlitre of dithiothreitol/NotI primer mix [0.5 μl of 1/25 diluted (dT)18 NotI adapter primer and 0.5 μl DTT per sample] and 2.5μl of first strand synthesis mix were added to each tube. Reactions were then incubated first at 37°C for 1 h, at 90°C for 5 min and finally on ice for 15 min. Samples were then diluted by adding 30 μl of water to each tube. The reverse transcribed samples were stored at -20°C until further use.

2.19 Cloning PCR products

The PCR-amplified products mixed with gel-loading dye were loaded in an agarose gel of appropriate strength along with molecular weight marker(s). Electrophoresis was conducted as described earlier allowing sufficient time for PCR bands to separate. The PCR products were viewed over a UV transilluminater and bands of predicted size were cut out of the gels.

The PCR bands were extracted from the agarose gels with glass milk and the Geneclean kit (Bio101, Anachem Ltd, Luton, Beds, UK) and stored frozen at -20°C. The cleaned DNA

samples (PCR bands) were blunt ended using the Klenow fragment of DNA polymerase 1 and then ligated to an EcoRV-digested Bluescript plasmid (pBSKII$^+$) using T4 DNA ligase. Alternatively, the PCR-amplified products were directly blunt ended with Klenow fragment and electrophoresed on normal agarose gel. The appropriate bands were cut out and purified with Geneclean kit before being ligated to pBSKII$^+$ at the EcoRV site using T4 ligase.

In-gel ligation was carried out with blunt-ended DNA run on low melting point NuSieve agarose gel. The DNA fragment from the appropriate size band was directly ligated to EcoRV-digested pBSKII$^+$ (10 ng) using T4 DNA ligase (Kovalic *et al.* 1991).

2.20 DNA sequencing

2.20.1 Plasmid template preparation

pBSKII$^+$ plasmid-infected *E.cColi* were used to produce template for sequencing. The DH5α strain of *E.coli* were inoculated into L-broth without antibiotics, and the culture was incubated overnight at 37°C oven with shaking. Competent cells were prepared and used for transformation of the pBSKII$^+$ with the ligated DNA fragment. The plasmid DNA containing the cloned DNA fragment was extracted by miniprep and purified using the Geneclean kit. Alternatively, purified miniprep DNA was prepared using QIAgen kit (supplied by Hybaid Ltd, Middlesex, UK).

2.20.2 Manual sequencing

2.20.2.1 Labelling reaction

Sequencing of single-stranded DNA templates was carried out according to the Sanger dideoxy termination method (Sanger et al. 1977) using thermosequenase sequencing kit according to the manufacturer's instruction.

2.20.2.1.1 Reagents

The reagents for the labelling reactions were universal primer, concentrated reaction buffer, 7deaza dGTP cycle mix, dCTP, thermosequenase enzyme, [α ^{35}S] dATP and miniprep extracts of the plasmid with the insert fragment.

2.20.2.1.2 Method

The universal primer (1µl), the concentrated buffer (2 µl), 7deaza dGTP (1 µl), dCTP (1 µl), water (5 µl), [α ^{35}S] dATP (0.5 µl) and thermosequenase enzyme (2 µl) were added to a 0.5 ml PCR tube for each reaction. The tubes prepared in this way were moved from the hood reserved for PCR and miniprep samples (5 µl) were added. The tubes were pulse spun to bring the contents to the bottom before being placed in a PCR machine. PCR amplification was carried out with the following parameters: 95°C for 15 s and 60°C for 30 s, 30-60 cycles.

2.20.2.2 Termination reaction

2.20.2.2.1 Reagents

The reagents were dGTP, dATP, dTTP, dCTP, stop solution (all provided in the kit) and PCR products.

2.20.2.2.2 Method

dGTP, dATP, dCTP and dTTP (all 4 µl) were pipetted into 0.5ml PCR tubes labelled G, A, C and T respectively. Reaction mix (3.5 µl) from the previous labelling step was added to each of these tubes and the contents were brought to the bottom of the tubes by pulse spinning. The tubes were placed in the PCR machine for 50 cycles and the reaction was stopped by the addition of stop solution (4 µl). The tubes were then heated to 70°C for 2min and loaded on an acrylamide gel or stored frozen at -20°C.

2.20.2.3 Poly acrylamide gel electrophoresis

2.20.2.3.1 Reagents

The reagents used for the preparation and running of polyacrylamide gel electrophoresis not included in the thermosequenase sequencing kit mentioned were:- 10% TBE; 0.5% TBE; fresh acrylamide solution [Ready made 40% 19:1 acrylamide/bis (15ml), 10%TBE (5 ml), urea (46 g) dissolved in water (30 ml) by heating in a beaker over magnetic stirrer and then making up to 100ml with water]; ammonium peroxodisulphate [ammonium peroxodisulphate (0.15 g) dissolved in of water (500 µl)]; TEMED (N,N,N',N'- Tetramethylethylene diamine from Sigma, Poole, Dorset, UK); acrylamide gel mix [acrylamide mix (70 ml), TEMED (90 µl), and ammonium peroxodisulphate (70 µl), mixed on a magnetic stirrer and immediately poured to cast a gel]; sodium phosphate (0.5 M); absolute alcohol; acetone; and dimethyl dichloro silane.

2.20.2.3.2 Preparation of the gel plate

Gel casting glass plates were wiped successively with water, absolute alcohol and then acetone. The smaller plate of the pair was wiped with 2% dimethyl dichlorosilane in 1,1,1-trichloroethane. White spacers were placed between plates and the plates were taped together tightly. The comb was cleaned with alcohol. The gel mix was poured in between the two plates using a sterile 10 ml syringe taking care that no bubbles formed. To avoid bubble formation, the plates were kept slightly slanting and the gel was introduced upwards from below. Excess gel was removed from the edges of the plates and the comb was inserted with the straight edge inside the gel. The assembly was left overnight under weights to allow the gel to fully set.

2.20.2.3.3 Loading the gel

Once the gel was set, the comb was removed, cleaned with alcohol and inserted back into gel with the comb teeth just touching gel. The tapes were removed and the gel assembly was fixed in a vertical electrophoresis apparatus (Bethesda Research Laboratories, Life Technologies

Ltd, UK). Running buffer (500 ml, 0.5× TBE) was poured into the top tank and running buffer (400 ml, 0.5× TBE) with sodium phosphate (80 ml, 0.5 M) was poured into the bottom tank. The wells in the gel were cleaned by flushing out any urea present with a Pasteur pipette. The plate assembly set was heated for 30 min. at about 90 volts, and the samples were heated at 70°C for 2 min. in a dry block before loading. Three microlitres each of G,A,T and C mixes of each sample were loaded using clean duck bill pipette tips fitted to the pipette. The first sample (all the four lanes) was loaded and the gel was run for a few minutes. The electric current was then stopped, and then the second sample was loaded after leaving an empty well in the gel in order to orient the gel after electrophoresis.

2.20.2.3.4 Developing the gel

After the samples had run the desired length in the gel, the power was switched off, the knobs were unscrewed to drain the buffer, the comb was removed and the plate assembly was removed. Whatman 3MM blotting paper was cut to the appropriate size and laid on the gel pressing evenly on the entire surface. The blotting paper was lifted gently from the plate, leaving the gel sticking to the paper. Saran (Kleanfilm) wrap paper, cut to the exact size as the blotting paper, was then laid over the gel. The gel attached to the blotting paper and saran wrap was vacuum dried on a flat gel drier. After drying (usually 2 h) the gel was removed and a little talcum powder was spread on to avoid stickiness. The gel was then placed in an X-OMATIC X-ray cassette (Kodak Scientific Imaging System through Anachem, Luton, Beds, UK) and fixed using adhesive tapes. A Kodak X-OMAT (XAR) film (Kodak Scientific Imaging System through Anachem, Luton, Beds, UK) was loaded when the cassette was in the dark room. The loaded cassette was left on the bench at room temperature for one or two days before being developed. X-ray film was developed automatically in a Xograph Compact X2 machine using Photosol XF-2 fixer and Photosol XD-2 developer (Xograph Ltd, Malmesbury, Wiltshire, UK).

2.20.3 Automatic Sequencing

Automatic sequencing was performed with a LiCor sequencer (MWG Biotech Ltd, Milton Keynes, UK).

2.20.3.1 Reagents

The reagents used for the preparation and running of polyacrylamide gel electrophoresis and the automated sequencing were 10mM EDTA, 10mM NaOH, 95% formamide, 0.01% Pararosniline (P7632, Sigma), long run buffer [(10 × TBE): 1340 mM Tris base, 450 mM boric acid, 25 mM Na_2 EDTA in water], thermosequenase kit [RPN 2438, Amersham], rapid gel XL [USB 75863, Amersham], chill out wax [999908, Biozym], DMSO [D8418, Sigma] and urea.

2.20.3.2 PCR reaction for sequencing

The reagents were thawed out in room temperature in a hood dedicated to PCR. For less than six samples, single tubes were used, whereas for more than six samples a microtitre plate was used. The tubes or the microtitre wells were labelled A, C, G, T and R. Master mix of 100 µM IRD labelled primer (1.5 µl), DMSO (1.4 µl) and water (16.1 µl) was added to each sample in the well marked R. The A, C, G and T kit reagents (1.5µl for each) were dispensed into the appropriately marked wells and the master mix (4.5 µl) from the wells marked R was added to all the wells. The miniprep DNA extract (2µl) (approximately 130 ng of the DNA insert per kilobase, according to manufacturer's instruction) was added to the appropriate wells. The contents of each well were mixed by pipetting up and down with separate filter pipette tips. Each filled well was overlaid with one drop of chill out wax and the microtitre plate was placed in the thermocycler PCR machine. PCR amplification was carried out with 15 cycles of 95°C melting, 57°C annealing, 68°C extension and a further 20 cycles of 95°C melting and 72°C annealing/extension. When the PCR was complete, the microtitre plate was taken out of the thermocycler and left wrapped in saran wrap at room temperature until denatured at 65°C for 5 min. The PCR products were loaded onto a gel for analysis.

2.20.3.3 Preparing acrylamide gel mix

The following reagents were mixed in a 250 ml thick-glass beaker using a magnetic stirrer without heating: urea (21 g), 10% long run TBE (5 ml), rapid gel excel (7.5 ml) (Amersham Lifescience) and water (28 ml). The urea-TBE- acrylamide mix was degased for >10 min using a vacuum pump. Just before casting, 500 µl of DMSO, 50 µl of TEMED and 350 µl of 10% Ammonium persulphate were added and mixed by very gentle whirling.

2.20.3.4 Preparation of the gel sandwich and pouring the gel

Both the glass plates (already cleaned with 10% SDS) were rinsed twice with water and absolute ethanol and dried with a white lint-free tissue. The glass plates and spacers were assembled with the rails. The acrylamide gel mix was sandwiched by pouring into the space between the plates while tapping them to ensure that no air bubbles formed. The well-former was put in place and tightened with the casting plate. The whole setting was wrapped in saran wrap to prevent drying up, and was left on the bench for 2 h for polymerisation. Once set, the casting bar and well-former were removed and the glass plate assembly was washed in water. Traces of acrylamide were cleaned from the outside of the glass plates by washing thoroughly with water. The glass plates were then dried.

2.20.3.5 Electrophoresis for automatic sequencing

The back plate, the upper and lower tanks and the comb of the sequencing machine were cleaned and dried. The upper buffer tank was slid into the gel assembly where the casting bar was taken out and the top set of screws were tightened to provide a good seal between the buffer tank and the glass plates. 1× TBE was poured to the upper buffer tank until the top of the meniscus reached the 'max fill' line. The remainder of 1 litre of 1% TBE was added to the lower tank taking care to avoid splashing the back plate. The upper and lower electrodes were attached along with the upper electrode cable and the instrument door was closed. The data

collection window was selected using the Base Image IR V4.0 icon and the appropriate gel configuration file was located. The window was minimized and the 'Create directory' option was selected from the 'File' menu. 'Focus' was then selected from the 'Options' menu on the scanner window to activate the automatic focus window. The 'Auto' button was selected to start the focusing routine. When focusing was complete 'Auto gain' option was selected in the 'Image' window from the 'Options' pull down menu. From the 'Parameters' menu in the scanner control window the 'Send' button was selected and the 'High Voltage' status and 'Scan' status options were selected. Electrophoresis was run until the temperature of the gel assembly reached 45°C before proceeding further.

Once the temperature reached 45°C the Li-Corr door was opened to remove the electrode from the upper buffer chamber. Using a 1 ml pipette the well was flushed to remove any urea which had leached out of the gel during the pre-run. A cleaned comb was inserted, to a depth of 1mm to form wells in the gel. A 8-channel glass syringe (multi-loading micropipette) was used to load the samples into the wells taking care not to allow lane leakage. The upper electrode was replaced and the door closed once the samples had all been loaded. When the display read 'Continue: Yes', the 'enter' button was pressed. The upper part of the image was deleted to reduce the amount of blank gel visualized, by selecting the 'Delete Image' option from the 'View' pull down menu in the image window. Electrophoresis was continued overnight and the computer screen was turned off during the run.

2.20.3.6 Image Analysis

The data file containing the sequence information was opened and transferred to the 'Image Analysis' file. The file was opened and the intensity of the image was adjusted so that the bands could be read. A, C, G and T lane definitions are shown separately for each sample. The 'Auto-single sample analysis' with 'Autostop' and 'Ambiguity Symbol' options were then selected. The sequence information was retrieved by pressing the 'Start' button. Only those sequences giving good stretch of unambiguous readings were accepted. The retrieved sequences were transferred to a report file and to the UNIX directory, using file transfer protocol (FTP).

2.20.4 Sequence analysis

The nucleotide sequences and deduced amino acid sequences were analysed using the Wisconsin UWGCG packages (Deverux *et al.* 1984).

2.21 Southern hybridization

2.21.1 Prehybridization

2.21.1.1 Reagents

1. Aqueous prehybridisation stock solution in water: SDS (7%), Na$_2$ EDTA (1 mM), and sodium phosphate at pH 7.4 (50 mM). This stock solution was stored in room temperature. 2. Aqueous prehybridisation (APH) working solution: salmon sperm DNA (5 mg/ml) was denatured by boiling at 95°C for 10min and immediately placing on ice for 2 min. The denatured salmon sperm DNA was added to the Aqueous Prehybridisation Stock Solution to a final concentration of 100 µg/ml.

2.21.1.2 Method

Prehybridization incubation was carried out in a hybridization oven (Biometra Ltd, Maidstone, Kent, UK). Nylon membranes with immobilized DNA were moistened in 6× SSC before transfer to a clean bottle containing pre-warmed APH working solution at a volume of 1 ml/10cm^2 membrane. Membranes were then incubated in the hybridisation oven with rotation for 2 h.

2.21.2 Hybridization

2.21.2.1 Reagents

1. Aqueous hybridisation (AH) stock solution in water: SDS (7%), Na_2 EDTA (1 mM), sodium phosphate at pH 7.4 (50 mM), poly ethylene glycol (10% w/v).

2. Working hybridization solution: Appropriate volumes of AH stock solution were warmed to the hybridisation temperature. The probe was prepared by random primed labelling with subsequent phenol-chloroform extraction and Sephadex column purification. The probe solution was denatured at 95°C for 3min and was immediately placed on ice for 2 min. The denatured probe was then immediately mixed into the warm AH stock solution to a minimum concentration of 1 million dpm/ml [10 ng/ml if the specific activity was of the order of 10^8 d.p.m./µg, or 2 ng/ml if it was of the order of 10^9 d.p.m./µg].

2.21.2.2 Method

On completion of prehybridisation, the APH working solution (with salmon sperm DNA) was poured off and immediately replaced with the working AH solution containing the denatured probe. Membranes were hybridised at an appropriate temperature, based on the calculated value of the melting temperature (Tm, details given in the appendix) overnight with constant rotation in the hybridisation oven.

2.21.3 Washing

Following hybridization, membranes were rinsed in 2 × SSC and taken through a series of 30 min washes. Low stringency washes began with 2× SSC, 0.1% SDS at 37°C. In order to increase the stringency of the washing, the temperature of washing was subjected small increments and the salt concentration (that is, SSC) was subjected to small decrements following the directions (Anderson 1995; Ausubel *et al.* 1998). For more details see the calculations in the appendix.

CHAPTER 3

ATTEMPTED PCR CLONING OF THE CHICKEN LEPTIN GENE

3.1 Introduction

The first paper describing the cloning of the mouse and human gene for the obese protein secreted by adipocytes (Zhang *et al.* 1994) presented a zooblot with a suggestion of a high degree of conservation of this gene across vertebrate phyla. The reverse transcription polymerase chain reaction (RT-PCR) technique (Innes *et al.* 1990) has successfully been employed to clone homologues from many other mammalian species including rat, rhesus monkey, domestic pig, sheep, cow, and the Israeli sand rat (*Psammomys obesus*) (Murakami and Shima 1995; Hotta *et al.* 1996; Bidwell *et al.* 1997; Dyer *et al.* 1997; Ji *et al.* 1998; Walder *et al.* 1997). The coding region of leptin gene is fairly conserved among these mammalian species (figure 3.1a, b). It therefore seemed likely that it should be possible to clone the chicken leptin gene by heterologous RT-PCR.

For heterologous RT-PCR cloning, degenerate oligonucleotide primers, a mixture of oligonucleotides varying in base sequence but with the same number of bases are substituted for specific sequence primers, resulting in the amplification of the heterologous gene sequence (Compton 1990). Degenerate oligonucleotide primers derived from the coding sequence of a protein can be used even if only a limited portion of the protein sequence is known. An extension of the same strategy may be applied when searching for new or uncharacterized sequences related to a known family of genes. An early example of this approach is the cloning of mammalian and avian members of the hepadna viruses using degenerate primers based on conserved regions of the amino acid sequence of viral reverse transcriptase (Mack and Sninsky 1988).

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horselepti >                                                                     GACACCAAAACCCTCATCAAGACGATTGTCACC  33
sheeplepti >          GTGCCCATCCGCAAGGTCCAGGATGACACCAAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACGATTGTCACC  57
gorillalep >          GTGCCCATCCAAAAAGTCCAGGATGACACCAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACAATTGTCACC   57
chipanzeel >          GTGCCCATCCAAAAAGTCCAGGATGACACCAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACAATTGTCACC   57
rhesuslept > ATGTATTGGAGAGAACCCTGTGGGGATTCTTGTGCGCTTTGGCCCTATCGTTCTACATCCAA   GCTGTGCCCATCCAAAAAGTACAAAGTGACACAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACAATTGTCACC  120
humanlepti > ATGCATTGGGGAACCCTGTGCGCGGATTCTTGTGGCTTTGGCCCTATCTTTTCTATGTCCAA   GCTGTGCCCATCCAAAAAGTCCAGGATGACACCAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACAATTGTCACC  120
ratleptinc > ATGTGCTGGAGACCCCTGTCCCGGTTCCGTTCCTGTGGCTTTGGTCCTATCGTTCTATGTTCAA  GCTGTGCCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTGTCACC  120
mouselepti > ATGTGCTGGAGACCCCTGTCCCGTCTGTCGGGCCGATTCCTGTGGCCCTATCGTTCTATGTTCAA  GCAGTGCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAAGACCATTGTCACC  120
pigleptinc > ATGCGCTGTGGACCCCTGTGCCCGTTGCCGATTCCTGTGGCCCTATCGTCCTACGTTGAA   GCCGTGCCCATCTGGAGAGTCCAGGATGACACCAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACGATTGTGAA  120
dogleptinc > ATGCGGTGTGGACCTCTGTGCCCGTTGCCGATTCCTGTGGCCCTATCGTCCTCCTACGTTGAA  GCTGTGCCAATCCGAAAAGTCCAGGATGACACCAAAACCCTCATCAAGACGATTGTTGAA  120
cowleptinc > ATGCGCTGTGGACCCCTGTATCGATTCCGATTCCTGTGGCCCTTTGGCCCTATCGTCTCTTATGCTTCAA  GCTGTGCCCATCCGCAAGTCCAGGATGACACCAAAAGTCCAAGATGACACCAAAACCCTCATCAAGACAATTGTCACC  120
CONSENSUS  > ATGcgcTGggGAcCcCTGTgccGaTTCcTGTGGCCTTTGGcCCTATCTgTccTatgTtcAa  GCtGTGCCcATCcaaAaAGTCCAggaTGACACCAAAAgTCCAagaTGACACCAAAACCCTCATCAAGACaATTGTcacc  120
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horselepti > AGGATCAATGACATTTCACACACGCAGTCAGTCTCCTCCAAACAGAGAGGTCACTGGTTG   GACTTCATTCCTGGCTTCACCCTGTCCTGAGTTGTCCAAGATGGCACCAGACATTGGCA   153
sheeplepti > AGGATCAATGACATCTCACACACGCAGTCCGTCGTCTCCTCCAAACAGAGAGGTCACTGGTTG  GACTTCATCCCTGGGCTCCACCCTCCTGAGTTGTCCAAGATGGCACCAGACATTGGCA   177
gorillalep > AGGATCACTGACATTTCACACACGCAGTCAGTCTCCTCCAAACAGAGAAGGTCACTGGTTG  GACTTCATTCCTGGGCTCCACCCATCCTGACCTTATCCAAGATGGCACCAGACACTGGCA   177
chipanzeel > AGGATCAATGACATTTCACACACGCAGTCAGTCAGTCTCCTCCAAACAGAGAAGGTCACCGGTTTG  GACTTCATTCCTGGGCTCCACCCATCCTGACCTTATCCAAGATGGCACCAGACACTGGCA  177
rhesuslept > AGGATCAATGACATTTCACACACGACGCGGTCGGTCTCCTCCAAACAGAGAGGTCACGGGTTG  GACTTCATTCCTGGGCTCCACCCGTCCTGACCTTATCCAAGATGGACCAGACACTGGCA  240
humanlepti > AGGATCAATGACATTTCACACACGCAGTCAGTCTCCTCCAAACAGAAAGTCACCGGTTG   GACTTCATTCCTGGGCTTCACCCTTATCCAAGATGGACCAGACACTGGCA  240
ratleptinc > AGGATCAATGACATTTCACACACGCAGTCGGTATCCGCCAGGCAGAGAGGCAGAGGGTCACGGGTTG  GACTTCATTCCTGGGCTTCACCCTCATTCTGAGTTTGTCCAAGATGGCACCAGACCCTGGCA  240
mouselepti > AGGATCAATGACATTTCACACACGCAGTCGGTATCCCGCCAAGCAGAGGGCAGAGGGTCACGGGTTG  GACTTCATTCCTGGGCTTCACCCATTCTGAGTTTGTCCAAGATGGCACCAGACCCTGGCA  240
pigleptinc > AGGATCAGTGACATTTCACACACGCAGTCGGTCTGTCTCCTCCAAACAGAGAGGTCACGGGTCTG  GACTTCATTCCTGGGCTCCACCCTCCTGAGTTTGTCCAAGATGGCACCAGACGTTGCC  240
dogleptinc > AGGATCAATGACATCTCACACACGCAGTCAGTCGGTCTCCTCCAAACAGAGAGGTCACTGGTTG  GACTTCATCCCTGGGCTCCACCCTCCTGAGTTTGTCCAAGATGGCACCAGACGCTGCG  240
cowleptinc > AGGATCAATGACATTTCACACACGCAGTCAGTCCGTCTCCTCCAAACAGGAGAGGTCACTGGTTG  GACTTCATCCCTGGGCTCCACCCTCCTGAGTTTGTCCAAGATGGCACCAGACATTGGCG  240
CONSENSUS  > AGGATCAaTGACATtTCACACACGCAGTcagTcgGTCTCCtCCAaaCAGaggTCACtGGtTG  GACTTCATtcCtGGGCtccAcCCytccTGAgtTTgTCcaaGAtGGCaCcAGACactGGcA  240
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horselepti > ATCTACCAACAGATCCTTACAAGTCTGCCTTCGACGATATCTGATCCAGATATCTAATGAC   CTGGAGAACCTCCGGGACCTTCTCCACCTGCTGGCCTCCTCCAAGAGTTGCCCCCTTGCCCC  273
sheeplepti > ATCTACCAACAGATCCTCACCAGTCTGCCGCTGCCTTCGACGTCTGATCCAAATATCTAATGAC  CTGGAGAACCTCCGGGACCTTCTCCACCTGCTGGCCTCCTCCAAGAGTGCCCCCTTGCCCG  297
gorillalep > GTCTACCAACAGATCCTCACCAGTATGCCTTCGACAAACATGATCCAAATATCCAACGAC   CTGGAGAACCTCCGGGACCTTCTCCACGTGCTGGCCTCCTCTAAGAGCTGCACTTGCCCC  297
chipanzeel > GTCTACCAACAGATCCTCACCAGTATGCCTTCGACAAACATGATCCAAATATCCAACGAC  CTGGAGAACCTCCGGGACCTTCTCCACGTGCTGGCCTCCTCTAAGAGCTGCACTTGCCCC  297
rhesuslept > ATCTACCAACAGATCCTCATCAATCTGCCTTCAATCGTGATCCAAATATCCAACGAC   TTGGAGAATCTCCGGGATCTTCTCCACGTGCTGGCCTCCTCTAAGAGCTGCCATTGCCCC  360
humanlepti > GTCTACCAACAGATCCTCACCAGTATGCCTTCCAGAAACGTGATCCAAATATCCAACGAC  CTGGAGAACCTCCGGGATCTTCTCCACGTGCTGGCCTCCTCTAAGAGCTGCCACTTGCCCC  360
ratleptinc > GTCTATCAACAGATCCTCACCAGTCTGCCTTCCCAAATGTGCTGCAGATAGTCCATGAC   CTGGAGAACCTGCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAGCTGCTCCCTGCCG  360
mouselepti > GTCTATCAACAGATCCTCACCAGTCTGCCTTCCCAAATGTGCTGCAGATAGTCCATGAC  CTGGAGAGATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAGCTGCTCCCCTGCCT  360
pigleptinc > ATCTACCAACAGATCCTCACCAGTCTGCTGCATTCCAGAAATGTGTCCAAATATCAATGAC   CTGGAGAACCTCCGGGACCTTCTCCACGTGCTGGCCTCCTCCAAGAGCTGCGCCCCTTGCCCC  360
dogleptinc > ATCTACCAACAGATCCTCACCAGTCTGCTTCCAGAAATGTGGTCCAAATATCTAATGAC  CTGGAGAACCTCCGGGACCTTCTCCACGTGCTGGCCTCCTCAAGAGCTGCCCCCTGCCCC  360
cowleptinc > ATCTACCAACAGATCCTCACCAGTCTGCCTTCGACAAATGTGGTCCAAATATCCAATGAC  CTGGAGAACCTCCGGGACCTTCTCCACCTGCTGCCGCCGCCGCCTCAAGAGCTGCCCCCTGCCCG  360
CONSENSUS  > aTCTAcCAACAgaTCCTcacCAgtcTgCcTTCcCaaAtgTgaTcCAaATAtCcaaTGAC   cTGGAGAAcCTcCGggAcCTtCTcCAccTGCTGgCcTcCTcctCCaAaGAGcTGCcccTGCCC  360
```

Figure 3.1a The conserved mammalian leptin coding region. The mammalian leptin sequences cloned by RT-PCR are compared with the mouse leptin coding region which was cloned by positional cloning (Zhang *et al.* 1994).

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```
horselepti > CAGGCCAGGGGTCTGGAGACCTTA
sheeplepti > CAGGTCAGGGCCCTGGAGAGCTTGGAGAGCCTGCGTCCTGGAAGCCTCCCTCTAC   TCCACCGAGGTGGTGGCCCGGCTACAGGGGTCTCTACAGGACAGTGTGCGGCAG  417
gorillalep > TGGGCCAGTGGCCTGGAGAGCTTGGACAGCCTGGGGGGTCCTGGAAGCTTCAGGCTAC   TCCACAGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTCAGGGGTCTCGTGGCAG  417
chipanzeel > TGGGCCAGTGGCCTGGAGAGCTTGGACAGCCTGGGGGGTGTCCTGGAAGCTTCAGGCTAC   TCCACAGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTCTGCAGGACACATGCTGTGGCAG  417
rhesuslept > TTGGCCAGTGGCCTGGAGACCTTGGAGACACGGGGTGTCCTGGAAGCTTCACTCTAC   TCCACGGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTCTGCAGGACATGCTGTGGCAG  480
humanlepti > TGGGCCAGTGGCCTGGAGACCTTGGACACCGGGGTGTCCTGGAAGCTTCAGGCTAC   TCCACAGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTCTGCAGGACATGCTGTGGCAG  480
ratleptinc > CAGACCCGTGGCCTGCAGAAGCCAGAGAGCCTCGCTCTAC   TCCACAGAGGTGGTGGCTCTGAGCAGGCTGCAGGGCTCTCTGCAGGACAATCTTCAACAG  480
mouselepti > CAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTCACTCTAC   TCCACAGAGGTGGTGGCTCTGAGCAGGCTGCAGGGCTCTCTGCAGGACAATCTTCAACAG  480
pigleptinc > CAGGCCAGGGCCCTGGAGACCTTGGAGAGCCTCCCTCTAC   TCCACAGAGGTGGTGGCTCTGAGCAGGCTGCAGGGCTCTCTGCAGGACACATGCTGCGGCAG  480
dogleptinc > CGGGCCAGGGGCCTGGAGACCTTTGAGAGCCTCCCTCTAC   TCCACGGAGGTGGTGGCCCTGAGCAGGCTGCAGGGGTCTCAGGGGTCTCGCGGCAG  480
cowleptinc > CAGTCAGGGCCCTGGAGAGCTTGGAGAGCCTGCGTCCTGGAAGCTTCCCTCTAC   TCCACAGAGGTGGTGGCTCTGAACAGACTGCAGGGGTCTCTGAACAGACATGCTTCGGCGG  480
CONSENSUS  > caGgcCaGtGgcCTGgAGAccttgGAgAGcCTcccTctaC   TCCACaGAGGTGGTGGCccTGAgcAgGcTgCAGGgtctcTgCAGGACATgcTgcggCag  480

sheeplepti > CTGGACCTCAGTCCCGGGTGCTGA  441
gorillalep > CTGGACCTCAGCCCTGGGTGCTGA  441
chipanzeel > CTGGACCTCAGCCCTGGGTGCTGA  441
rhesuslept > CTGGACCTCAGCCCTGGGTGCTGA  504
humanlepti > CTGGACCTCAGCCCTGGGTGCTGA  504
ratleptinc > TTGGACCTTAGCCCTGAATGCTGA  504
mouselepti > TTGGATGTTAGCCCTGAATGCTGA  504
pigleptinc > CTGGACCTCAGCCCTGGCTGCTGA  504
dogleptinc > CTGGACCTCAGCCCTGGGTGCTGA  504
cowleptinc > CTGGACCTCAGTCCCGGGGTGCTGA  504
CONSENSUS  > cTGGAccTcAGccCtGggTGCTGA  504
```

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Figure 3.1a The conserved mammalian leptin coding region. The mammalian leptin sequences cloned by RT-PCR are compared with the mouse leptin coding region which was cloned by positional cloning (Zhang *et al.* 1994).

```
     mouseleptincd  MCWRPLCRFL WLWSYLSYVQ AVPIQKVQDD TKTLIKTIVT RINDISHTQS VSAKQRVTGL DFIPGLHPIL SLSKMDQTLA VYQQVLTSLP SQNVLQIAND
       ratleptincd  MCWRPLCRFL WLWSYLSYVQ AVPIHKVQDD TKTLIKTIVT RINDISHTQS VSARQRVTGL DFIPGLHPIL SLSKMDQTLA VYQQILTSLP SQNVLQIAHD
  chipanzeeleptincd  ~~~~~~~~~~ ~~~~~~~~~~ ~VPIQKVQDD TKTLIKTIVT RINDISHTQS VSSKQKVTGL DFIPGLHPIL TLSKMDQTLA VYQQILTSMP SRNMIQISND
      humanleptincd  MHWGTLCGFL WLWPYLFYVQ AVPIQKVQDD TKTLIKTIVT RINDISHTQS VSSKQKVTGL DFIPGLHPIL TLSKMDQTLA VYQQILTSMP SRNVIQISND
    gorillaleptincd  ~~~~~~~~~~ WLWPYLFYVQ ~VPIQKVQDD TKTLIKTIVT RISDISHTQS VSSKQKVTGL DFIPGLHPIL TLSKMDQTLA VYQQILTSMP SRNMIQISND
     rhesusleptincd  MYWRTLWGFL WLWPYLFYIQ AVPIQKVQSD TKTLIKTIVT RINDISHTQS VSSKQRVTGL DFIPGLHPVL TLSQMDQTLA IYQQILINLP SRNVIQISND
        cowleptincd  MRCGPLYRFL WLWPYLSYVE AVPIRKVQDD TKTLIKTIVT RINDISHTQS VSSKQRVTGL DFIPGLHPLL SLSKMDQTLA IYQQILASLP SRNVIQISND
      sheepleptincd  ~~~~~~~~~~ ~~~~~~~~~~ ~VPIRKVQDD TKTLIKTIVT RINDISHTQS VSSKQRVTGL DFIPGLHPLL SLSKMDQTLA IYQQILASLP SRNVIQISND
      horseleptincd  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~D TKTLIKTIVT RINDISHTQS VSSKQRVTGL DFIPGLHPLL SLSKMDQTLA IYQQILTSLP SRNVIQISND
        pigleptincd  MRCGPLCRFL WLWPYLSYVE AVPIWRVQDD TKTLIKTIVT RISDISHMQS VSSKQRVTGL DFIPGLHPVL SLSKMDQTLA IYQQILTSLP SRNVIQISND
        dogleptincd  MRCGPLCRFL WLWPYLSCVE AVPIRKVQDD TKTLIKTIVA RINDISHTQS VSSKQRVAGL DFIPGLQPVL SLSRMDQTLA IYQQILNSLH SRNVVQISND

101                                                 150
     mouseleptincd  LENLRDLLHL LAFSKSCSLP QTSGLQKPES LDGVLEASLY STEVALSRL QGSLQDILQQ LDVSPEC*
       ratleptincd  LENLRDLLHL LAFSKSCSLP QTRGLQKPES LDGVLEASLY STEVALSRL QGSLQDILQQ LDLSPEC*
  chipanzeeleptincd  LENLRDLLHV LAFSKSCHLP WASGLETLDS LGGVLEASGY STEVALSRL QGSLQDMLWQ LDLSPGC*
      humanleptincd  LENLRDLLHV LAFSKSCHLP WASGLETLDS LGGVLEASGY STEVALSRL QGSLQDMLWQ LDLSPGC*
    gorillaleptincd  LENLRDLLHV LAFSKSCHLP WASGLETLDS LGGVLEASGY STEVALSRL QGSLQDMLWQ LDLSPGC*
     rhesusleptincd  LENLRDLLHL LAFSKSCHLP LASGLETLES LGDVLEASLY STEVALSRL QGSLQDMLWQ LDLSPGC*
        cowleptincd  LENLRDLLHL LAASKSCPLP QVRALESLES LGVVLEASLY STEVALSRL QGSLQDMLRQ LDLSPGC*
      sheepleptincd  LENLRDLLHL LAASKSCPLP QVRALESLES LGVVLEASLY STEVALSRL QGSLQDMLRQ LDLSPGC*
      horseleptincd  LENLRDLLHL LASSKSCPLP QARGLETL~~ ~~~~~~~~~~ ~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~
        pigleptincd  LENLRDLLHL LASSKSCPLP QARALETLES LGGVLEASLY STEVALSRL QGALQDMLRQ LDLSPGC*
        dogleptincd  LENLRDLLHL LASSKSCPLP RARGLETFES LGGVLEASLY STEVALNRL QAALQDMLRR LDLSPGC*
```

Figure 3.1b The conserved mammalian leptin coding region. The published mammalian leptin amino acid sequences are compared.

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During the course of research done for this thesis (August 1997), a putative chicken leptin cDNA sequence was published in Genbank sharing < 70% identical base pairs with the mouse cDNA, which was later (October 1997) updated with another cDNA sequence (Genbank accession: AF012727) that is 95% identical to the mouse leptin coding region at the nucleotide level. According to the authors (Taouis *et al.* 1998) the latter sequence was obtained from adipose tissue and liver RNA of broiler chicken by RT-PCR. An almost identical sequence (with a single difference over 503 nucleotides) was also reported by another group (Ashwell *et al.* 1999), again using the RT-PCR method.

The aim of these experiments was therefore, firstly to isolate an avian homologue of leptin and secondly, after the publication of Taouis *et al.* (1998) and Ashwell *et al.* (1999a) to confirm their findings using primers designed from the published sequence.

3.1.1 Research approach

RT-PCR primers were designed based on conserved mammalian leptin gene sequences and degenerate oligonucleotide sequences based on the mouse leptin gene.

These primers were used in combination with chicken genomic DNA (gDNA) or complementary DNA (cDNA) from chicken adipose tissue as templates.

Using these primers and templates, an extended series of experiments were conducted with modified/optimized reaction conditions to take into account the possibility of poor homology between mammalian and avian leptin gene sequences.

In order to confirm the putative chicken leptin cDNA sequences described by Taouis *et al.* (1998) and Ashwell *et al.* (1999a), fat and liver total RNA samples extracted from different strains of chicken were subjected to RT-PCR, using primers based on the chicken leptin sequence reported by these authors. These were used to attempt RT-PCR cloning over a wide range of reaction conditions.

3.2. Methods

3.2.1 Experimental animals and tissues

Adult male and female C57BL6 mice, Scottish black face sheep and broiler and bantam chickens from Roslin Institute stock were used for extraction of total RNA and genomic DNA. Total RNA was extracted from the abdominal fat pad and mesenteric fat (adipose tissue) and the liver. Genomic DNA was extracted from chicken whole blood and various tissues of mice and sheep. All animals were *ad libitum* fed.

3.2.2 General reaction conditions

A typical RT-PCR reaction was performed in 0.5ml PCR tubes in a total volume of 25µl and final concentrations of $1 \times$ magnesium-free PCR buffer, 3 mM $MgCl_2$, 200µM each dNTP, and 1 µM of each primer together with 1 unit of Taq polymerase. Common mixes were prepared wherever possible to reduce pipetting and to minimize contamination. The template DNA (gDNA or cDNA) was added last, in a tissue culture hood in an adjacent laboratory, away from the dedicated area where the other procedures were carried out. After the addition of the template, the tubes were spun briefly in a microfuge and immediately placed in the thermal cycler, which was preheated to 94°C to reduce the chances of mispriming at low temperatures. Denaturation, annealing and extension temperatures were 94°C, 57°C and 72°C respectively. Times used were 15 s denaturation, 30 s annealing and 45 s extension. A Hybaid Omnigene thermal cycler with heated lids was used to prevent evaporation and condensation of the contents of the tubes.

Negative controls in which the template was omitted were always included. Reactions were repeated in separate tubes with chicken glyceraldehyde phosphate dehydrogenase (GAPDH) primers (table 3.1) as positive controls. During the conduct of the PCR

reaction, simultaneous amplification of mammalian leptin templates was usually avoided in order to prevent cross contamination. However, towards the end of these series of experiments, PCR amplification of the mouse leptin sequence was successfully carried out to serve as a positive control.

PCR products were visualized by running on 1-3% agarose gel containing ethidium bromide. If appropriate, the visualized bands were further characterized by Southern analysis with mouse leptin probes, or by subcloning and sequencing. For cloning, the PCR product was blunt-ended with the Klenow fragment of DNA polymerase I, and after gel electrophoresis, the target DNA band was purified using a Geneclean kit (Bio101), and ligated into pBSKII$^+$. This was further transformed into *E.coli* XL1blue (see chapter 2).

If the PCR product was to be used as a template in a second PCR, the PCR mix was run on a 1% Nuseive gel and the bands were cut out. The DNA in the cut band was diluted in water and used as template for further PCR using one new single primer only ("hemi-nested") or with two new primers ("nested"). This procedure was carried out to verify the specificity of the amplified sequence since it should contain the sequence of interest within it. If the amplified band represents the sequence predicted, amplification with a nested primer should produce a product. A negative result indicates that the sequence is not that predicted. This method was widely used in the attempts to amplify the published chicken leptin sequence.

For some experiments where common primers to the mouse and the putative chicken leptin sequences were used, the RT-PCR products were run on a 3% agarose gel and were blotted to positively charged nylon membrane (Hybond N+, Amersham) using alkaline transfer buffer. The Southern blotted nylon membrane was later used for hybridizing with the mouse leptin probe (as in figure 3.5) in order to confirm the sequence identity of the amplified bands.

3.2.3 Design of primers

The programme 'Prime' of UWGCG was used to select oligonucleotide primers from the consensus mammalian leptin sequence (see figure 3.1) as the template DNA sequence. 'Prime' analyzes a template DNA sequence and chooses primer pairs for PCR considering a variety of constraints on the primer and amplified product sequences. The programme uses the annealing test to check individual primers for self-complementarity and to check the two primers in a PCR primer-pair for complementarity to each other and also screens against non-specific primer binding on the template sequence. The range of primer size, the difference in primer melting temperatures and the range of product sizes were set to the default values of the programme.

Fourteen primers (table 3.1) were designed and used in different combinations. The position of these primers relative to the mouse leptin sequence is shown in figure 3.2. When designing these primers, the entire mammalian leptin coding region was taken into consideration. Some of these primers (primers RF2, RR1, RR2, RR3, and the degenerate RF3) diverge from the mouse sequence by one or two nucleotides because they were based on a consensus sequence of mammalian species (see figure 3.1). An anchored oligo (dT) 18 adaptor primer was also used, which was included in the sequence of the primer used for reverse transcription. Lyophilised primers were reconstituted according to the manufacturer's instructions in water to a concentration of 100 pmol/μl and stored in 25 μl aliquots at -20°C.

3.2.4 Optimization of PCR conditions

The PCR reactions were carried out as described using the standard conditions or modified by titrating with $MgCl_2$ ranging from 0.5 to 5 mM at 0.5 mM intervals, but mostly at 1, 3 and 5mM; annealing at 50°C to 60°C, but mostly at 52°C and 57°C and using touch-down conditions (Don *et al.* 1991; Roux and Hecker 1997) for PCR amplification. In touch-down PCR the annealing temperature was reduced by 0.5°C in successive one-cycle steps from 64°C to 60°C, or from 60°C to 56°C. Once a temperature of 60°C or 56°C was reached, a further 30 cycles were run under the same conditions. The extension time for the final cycle was 5 min.

Sl No.	Primer	Sequence	Sense	A	C	G	T	A+T	G+C	Total	G+C%	Tm	Ta
1	2G7for	CCA GGG AGG AAA ATG TG	Forward	6	2	7	2	8	9	17	52.9	52	47
2	ob1for	GCA GTG CCT ATC CAG AAA GTC CA	Forward	7	7	5	4	11	12	23	52.2	70	65
3	ob4for:	CCC TCA TCA AGA CCA TTG TCA	Forward	6	8	2	5	11	10	21	47.6	62	57
4	RFl	TAT CCA CAA AGT CCA GGA TGA	Forward	8	5	4	4	12	9	21	42.9	60	55
5	RF2	AGT CCA GGA TGA CAC CAA AAC	Forward	9	6	4	2	11	10	21	47.6	62	57
6	RF3	GTC ACC AGG ATC AAY GAY AT	Forward	7	4	4	3	10	8	18	44.4	52	47
7	RF4	CAC TGG CTT GGA CTT CAT TC	Forward	3	6	4	7	10	10	20	50.0	60	55
8	2G7rev	CAT CCT GGA CTT TCT GGA TAG G	Reverse	4	5	6	7	11	11	22	50.0	66	61
9	Ob1rev	CTG CTC AAA GCC ACC ACC TCT GT	Reverse	5	9	3	5	10	12	22	54.5	68	63
10	ob4rev	AAG AAT GTC CTG CAG AGA GCC	Reverse	7	5	6	3	10	11	21	52.4	64	59
11	RR1	GTG GAG TAG AGC GAG GCT TC	Reverse	4	3	9	4	8	12	20	60.0	64	59
12	RR2	CTC AGA GCC ACC ACC TCT GT	Reverse	4	9	3	4	8	12	20	60.0	64	59
13	RR3	GCC AGT GTC TGG TCC AT	Reverse	2	5	5	5	7	10	17	58.8	54	49
14	RR4	ATG TCC TGC AGA GAG CCC TG	Reverse	4	6	6	4	8	12	20	60.0	64	59
15	Mob1	CAG ATT CGA TAT GGC TTA ATG GG	Forward	6	3	7	7	13	10	23	43.47	66	61
16	MLep4	CAG GGC TCT CTG CAG GAC AT	Reverse	4	6	6	4	8	12	20	60.0	64	59
17	GAPDH-for	AGT CAT CCC TGA GCT GAA TG	Forward	5	5	5	5	10	10	20	50.0	60	55
18	GAPDH-rev	ACC ATC AAG TCC ACA CG	Reverse	6	7	2	2	8	9	17	52.9	52	47

Table 3-1 Primers used in the PCR. The forward (sense) primers are suffixed with for or designated as RF and the reverse (antisense) primers are suffixed with rev or designated as RR. Also shown the chicken GAPDH primers used to amplify the templates as positive controls. The annealing temperature (T_a) can be calculated from the melting temperature (T_m) of the primers using the equation (Suggs et al. 1981): $T_a = T_m - 5°C = 2 (A+T) + 4 (G+C) - 5°C$

70

Mouse   1 aqqqatccctqctccaqcaqctqcaaqqtqcaaqaaqaaqaaqatcccagqqqaqqaaa 58
            Taouis-for →   2G7for

Mouse   59 ATGTGCTGGAGACCCCTGTGTCGGTTCCTGTGGCTTTGGTCCTATCTGTC 108
Chicken   1 --------------------------------A----------T--C---TGT 41

                        ob1for → RF1   RF2 →
                                     2G7rev ←
Mouse   107 TTATGTTCAAGCAGTGCCTATCCAGAAAGTCCAGGATGACACCAAAACCC 158
Chicken   42 --------------------GTG-----TCT------------------------ 91

                        ob4for   RF3 →
Mouse   159 TCATCAAGACCATTGTCACCAGGATCAATGACATTTCACACACGCAGTCG 208
Chicken   92 -- 138

                              RF4 →
Mouse   209 GTATCCGCCAAGCAGAGGGTCACTGGCTTGGACTTCATTCCTGGGCTTCA 258
Chicken   139 -- 188

                              RR3 ←
Mouse   259 CCCCATTCTGAGTTTGTCCAAGATGGACCAGACTCTGGCAGTCTATCAAC 308
Chicken   189 -- 238

Mouse   309 AGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCAGATAGCCAATGAC 358
Chicken   239 -- 288

Mouse   359 CTGGAGAATCTCCGAGACCTGCTCCATCTGCTGGCCTTCTCCAAGAGCTG 408
Chicken   289 -- 338

Mouse   409 CTCCCTGCCTCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCG 458
Chicken   339 -- 388

                        RR1 ←   ob1rev & RR2 ←
Mouse   459 TCCTGGAAGCCTCACTCTACTCCACAGAGGTGGTGGCTTTGAGCAGGCTG 508
Chicken   389 -- 438

                        RR4 ←   ob4rev ←
Mouse   509 CAGGGCTCTCTGCAGGACATTCTTCAACAGTTGGATGTTAGCCCCTGAATG 558
Chicken   439 ------------------------------------A--------G----- 488

                              Taouis-rev ←
Mouse   559 CTGAaqtttcaaaggccaccaggctcccaagaatcatqtaqagggaagaa 609
Chicken   489 ---- 492

Fig. 3.2. Diagram showing the positioning of the primers used. The positions are relative to the mouse sequence (Zhang *et al.* 1994; accession number U18812). Nucleotides different from the published chicken leptin sequence (accession number AF012727) are indicated at the bottom of each line. The lines (-) represent nucleic acid identity. Capital letters represent coding sequence. Lowercase letters represent flanking, noncoding sequences. Overlapping primers (e.g.ob1for and RF1, ob1rev and RR2) reflect the strategy of designing primers to the most conserved regions the of mammalian leptin genes. Some of these primers (primers RF2, RR2, RR1, RR3 and the degenerate RE3) diverge from the mouse sequence by one or two nucleotides since they were made according to a consensus of mammalian sequences (see figure 3.1). The rest of the primers are identical to the mouse sequence and some of them are also identical to the published chicken sequence (e.g. ob4for, ob4rev, RF4, RR4). The primers used by Taouis *et al.* (1998) to clone the published chicken leptin sequence, which flank the untranslated regions, are also illustrated. Note that the Taouis-for primer extends into the untranslated region by a further 8 bases. However, the first 7 of these 8 bases do not match the corresponding mouse sequence (accession numbers U36238, U52147).   71

3.2.5 Analysis of cloned sequences

The cloned fragments were sequenced and analysed using the Wisconsin GCG package (version 10), first to locate the position of the primers used and the flanking vector sequences, and then to check homology between the fragments. Subsequently, the vector sequences were edited out and the remaining sequences were analysed using BLAST at the National Centre for Biological Information (NCBI) world wide web site (http://www.ncbi.nlm.nih.gov/blast/) in order to compare the query sequence with possible homologues in the Genbank. Special attention was given to the identification of open reading frames and exon-intron boundaries from the annotations given in the published sequences. If homology existed between the PCR sequences and known sequences in the database, then a check was made for an open reading frame. In case of genomic PCR products, intron-exon boundaries were checked and it was also verified whether the protein coded displayed any homology.

3.3 Results

3.3.1 Attempts to amplify a leptin cDNA homologue from chicken adipose tissue cDNA

Amplification was performed on reverse transcribed total RNA from chicken adipose tissue (prepared as detailed in chapter 2). Numerous combinations of the primers were tried with a range of conditions. However, only one combination produced bands which corresponded with the expected product size. A band of ~378bp was generated with primers RF1 and RR2 using between 1mM and 3mM Mg^{++} annealed at 57°C and 40 cycles (figure 3.3). This was of the size predicted from mammalian sequence. The experiment was repeated once again with consistent results. Analysis of the amplified DNA by cloning and sequencing indicated that there was no sequence which had homology with any known gene in the database (see table 3.2a, b).
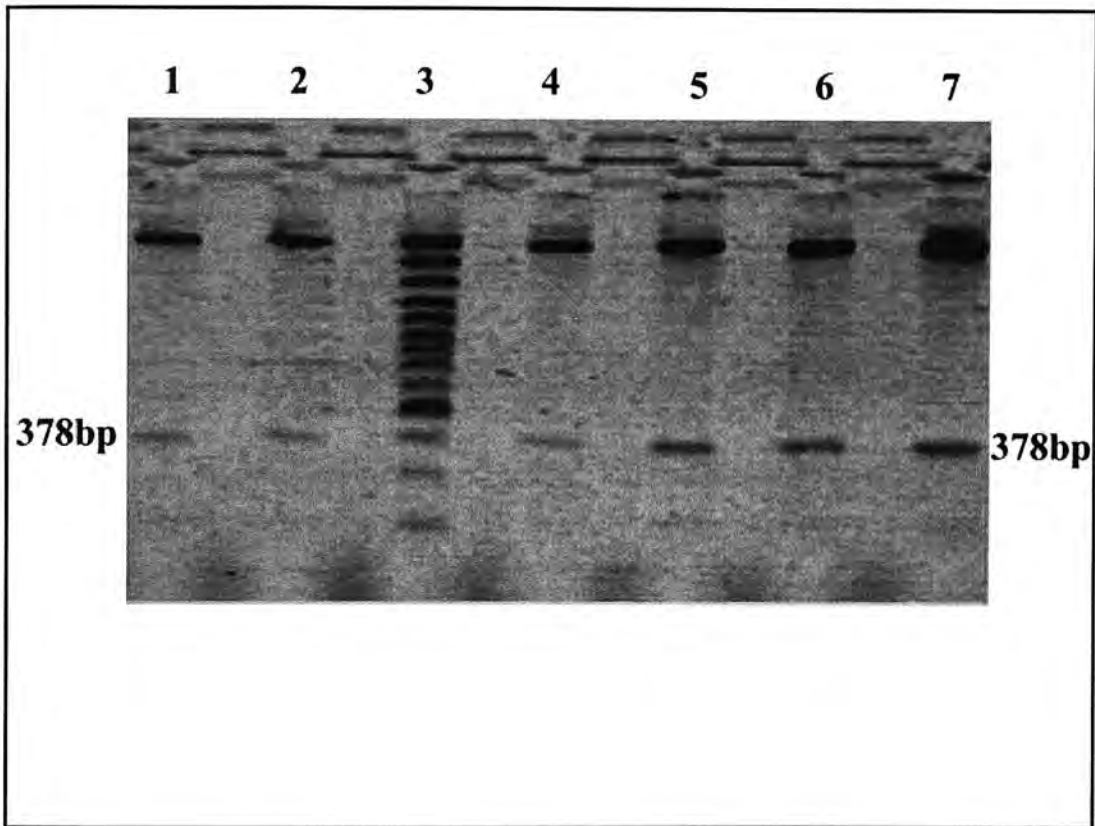
Figure 3.3:The 378bp band generated by RT-PCR with primers RF1 and RR2. An expected band of ~378bp was generated from chicken abdominal fat RNA by RT-PCR with primers RF1 and RR2 at 0.5 mM to 3 mM Mg^{++}, annealing temperature of 57°C after 40 cycles of PCR. Later, the DNA band was cloned in Blue script plasmid. Shown in the figure are the ~378bp band amplified in all the lanes. The quantity increases with increasing amount of Mg++ from 0.5mM to 3.0mM with 0.5 mM in each step (lanes 1, 2 and 4-7). The sequence obtained after cloning of the fragment proved to be unrelated to mammalian leptin sequence (table 3.3). Lane 3 shows the 100bp ladder plus (MBI Fermentas, Vilnius, Lithuania through Helena Biosciences Ltd., UK) used as the DNA size marker.

Serial No. RT-PCR	Clone(s): 1Or, 4-18, 5-I and 7-vi
Fragment size: ~378base pairs	Sequence length: 535 bases

Consensus with the contig fragment assembly

```
5       smpl4-18-ed                    <----+
   4       smpll-or-ed             +--------->
   3       smpl5-i-ed        <---------+
   2       smpl7-vi-ed +------->
   C       CONSENSUS   +------------------------->
                       |----------|----------|----------|---------|---------|
                       0         200        400        600       800
```

Consensus sequence

```
  1    CTGATTCACA GCCACCACCT ATGTCTAGGA GATTCCTAAC CCACAATTAA GGGCATAGTT
 61    GTAATACCAA ACACACCCAG ATGTCAGCAT GTCTGGAGAA AAAGCAGAGC ACCTATATGA
121    GCAGTTCAGC TskCAtCACr sTACwCtAGy AgCAACACAA CATCACATGC AAACAACCAG
181    CATACTGTTA TTAAATGTTC ACAAAGCTCA GCTGGAAATA AAGGTATGTA TAATTTTAGC
241    AGTGTCTAAT TAAGTTCCAT GTCTTTAAAA CACCTTACCC CACTGTAGCC AACATTACAC
301    AGATGTGGTr ksATCAAGCy ACCACATATG TTCCAGGAGA GACTCAAGAG TACTCTTGTA
361    AAATGCATGA AGATCTATTA CGGATTCAAA ACCTCTTTGG GATAAGGAAG GGGATCAGAA
421    TGCATCTTAA ATTAGCATGT CTGTGTmwCT ArrATcarAs yTmAGwwmCy yTmCTsyTCm
481    TcCAwCAGrw AGCCTTGTCC TCTTTACCTC TCACAGAGGT GGTGGCTCTG AATCAA
```

Homology

Hits	Sequence	Length (bp)	Extent
45	Homo sapiens, clone hRPK.17_A_1	144,260	22/22
	Canis familiaris mRNA for leptin	2,925	21/21
	Homo sapiens WD repeat domain 3 (WDR3), mRNA	3,850	19/19
	Homo sapiens BAC clone RP11-512E16 from 7p11.2-p21	151,316	22/23

Table 3.2a: The consensus sequence of the chicken RT-PCR. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained from the sequences amplified from chicken adipose tissue cDNA with primers RF1 and RR2 (as in figure 3.3). Other leptin sequences are not included in the list since it represents one of the primers used. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambiguous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No. RT-PCR	Clone(s): 1Or, 4-18, 5-I and 7-vi
Fragment size: ~378base pairs	Sequence length: 535 bases

Possible translations

1. LIHSHHLCLGDS*PTIKGIVVIPNTPRCQHVWRKSRAPI*AVQLXSXYXSSNTTSHANNQHTVIKCSQSS AGNKGMYNFSSV*LSSMSLKHLTPL*PTLHRCGXIKXPHMFQERLKSTLVKCMKIYYGFKTSLG*GRGSE CILN*HVCVXXIXXXXXXXSSXRXPCPLYLSQRWWL*I

2. *FTATTYV*EIPNPQLRA*L*YQTHPDVSMSGEKAEHLYEQFSXHHXTLXATQHHMQTTSILLLNVHKAQ LEIKVCIILAVSN*VPCL*NTLPHCSQHYTDVVXSSXHICSRRDSRVLL*NA*RSITDSKPLWDKEGDQN AS*ISMSVXLXSXXXXXXXXPXXSLVLFTSHRGGGSES

3. DSQPPPMSRRFLTHN*GHSCNTKHTQMSACLEKKQSTYMSSSAXITXX*XQHNITCKQPAYCY*MFTKLS WK*RYV*F*QCLIKFHVFKTPYPTVANITQMWXXQATTYVPGETQEYSCKMHEDLLRIQNLFGIRKGIRM HLKLACLCX*XQXXXXXLXXXQXALSSLPLTEVVALNQ

4. LIQSHHLCER*RGQGXLXXEXXXXXXLILXTQTC*FKMHSDPLPYPKEVLNP**IFMHFTRVLLSLSWNI CGXLXXPHLCNVGYSGVRCFKDMELN*TLLKLYIPLFPAELCEHLITVCWLFACDVVLLLXXXDXS*TAH IGALLFLQTC*HLGVFGITTMPLIVG*ESPRHRWWL*I

5. *FRATTSVRGKEDKAXXWXXXXXXXX*X*XHRHANLRCILIPFLIPKRF*IRNRSSCILQEYS*VSPGTY VVA*XXHICVMLATVG*GVLKTWNLIRHC*NYTYLYFQLSFVNI**QYAGCLHVMLCCX*XXVMXAELLI *VLCFFSRHADIWVCLVLQLCP*LWVRNLLDIGGGCES

6. DSEPPPL*EVKRTRLXXGXXXXXLXXDXXXTDMLI*DAF*SPSLSQRGFESVIDLHAFYKSTLESLLEHM WXLDXTTSV*CWLQWGKVF*RHGT*LDTAKIIHTFISS*AL*TFNNSMLVVCM*CCVAXXVX*XXLNCSY RCSAFSPDMLTSGCVWYYNYALNCGLGIS*T*VVAVNQ

Homology

Hits	Sequence	Length (bp)	Extent
	No homology found		

Table 3.2b: The consensus sequence of the chicken RT-PCR translated in all possible six frames and their homology. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained from the sequences amplified from chicken adipose tissue cDNA with primers RF1 and RR2 (as in figure 3.3). The sequence was compared with Swissprot protein database using the Blastx function in the NCBI with an option for 500 alignments to be displayed. However, this sequence did not have any match in the database.

The ~378bp band DNA from primers RF1 and RR2 was used in a nested PCR with other primers. Nesting of this DNA with primers RF1 and RR3 generated a conspicuous band of ~170bp at an annealing temperature of 57°C and 2mM $MgCl_2$, which is the size predicted from the mouse sequence (figure 3.4). When the DNA from this band was cloned and sequenced, it had no similarity with published leptin sequences (table 3.3a-c).

Comparison of the sequences with the Swissprot database with a blastx function of NCBI did not reveal any similarity with known proteins or any functional domains within the proteins.

The negative controls consisting of water instead of template DNA and all other PCR reagents in most of the cases produced no bands at all. Wherever amplification bands were visible in blank controls, all the PCR products were discarded and the reactions were repeated with fresh reagents. All batches of chicken cDNAs were verified as templates to amplify chicken GAPDH sequence using the chicken GAPDH primers (table 3.1) as positive control. Also, towards the end of these PCR experiments, all sets of primers were shown to be amplifying predicted size bands from the mouse cDNA templates.

3.3.2 Attempts to amplify a leptin gene homologue from chicken gDNA

Amplification of chicken genomic DNA with primers RF1 and RR2 using 3mM Mg^{++} and an annealing temperature of 55°C, yielded high and low molecular weight bands of ~2kb and 1.2kb respectively (figure 3.5). When the primers RF1 and RR3 were used, with the same Mg^{++} concentration and annealing and cycling conditions, five bands of approximately 1.5, 0.9, 0.7, 0.5 and 0.4kb were generated. Unfortunately, these five bands could not be reproduced consistently in confirmatory experiments. Attention was therefore focused on the 2kb and 1.2kb PCR bands obtained with primers RF1 and RR2. These were nested and hemi-nested with primer RR3 and the degenerate primer RF3 (table 3.1). No amplification was obtained from the 2kb band but the 1.2kb band yielded two new bands of 950bp and 850bp (figure 3.6). This was within the expected size range

76
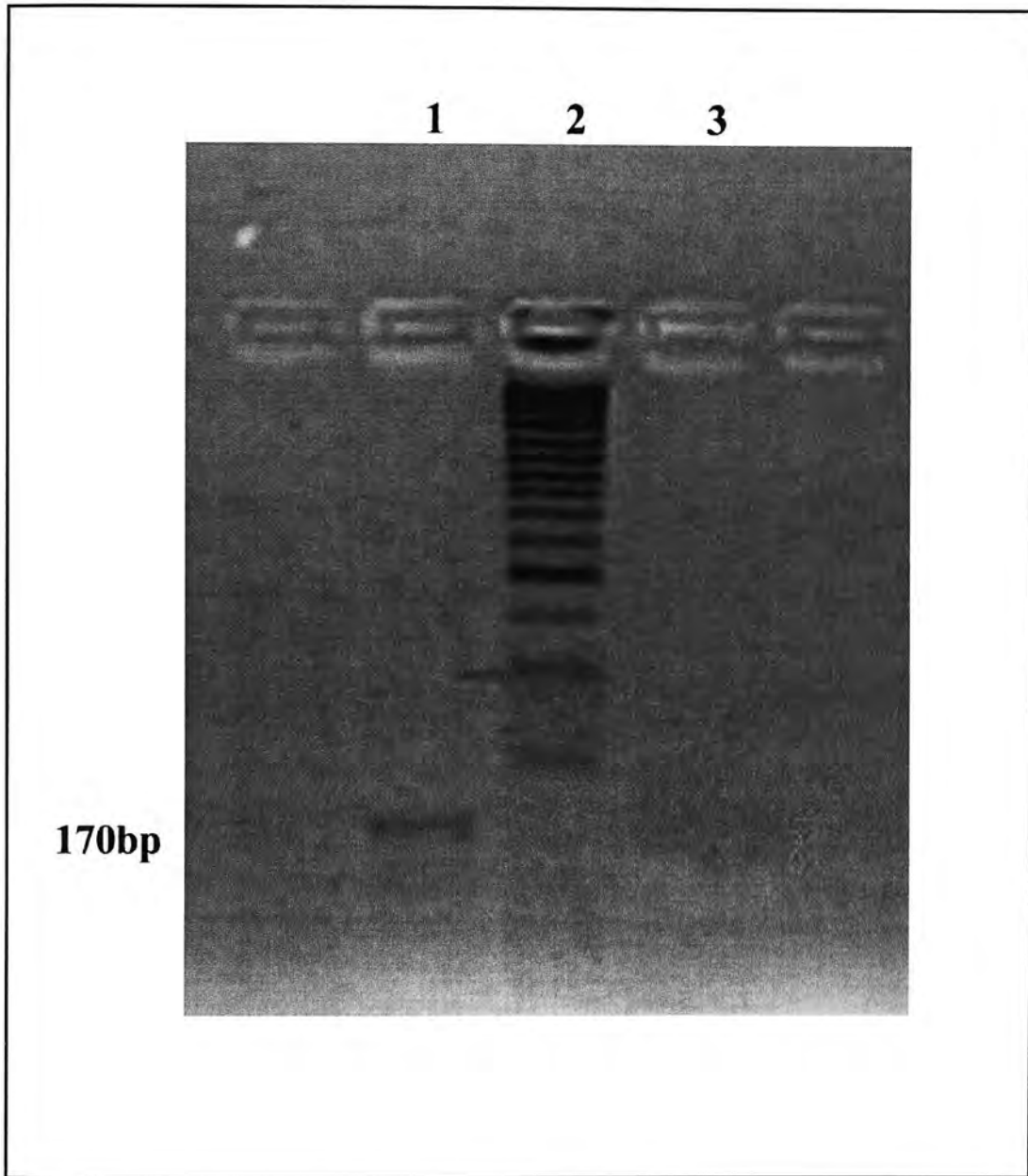
Figure 3.4: The nested RT-PCR of the ~378bp band generated with primers RF1 and RR2 using primers RF1 and RR3. The 378bp band DNA from RF1 and RR2 as in Figure 3.3 was used to nest/hemi-nest with the other primers. Nesting of this DNA with primers RF1 and RR3 generated a conspicuous band of the predicted size at 170bp (lane1) at 57°C annealing temperature and 2mM MgCl$_2$, consistent with that predicted from mammalian sequence. Lane 2 is the 100bp ladder plus (MBI Fermentas, Vilnius, Lithuania through Helena Biosciences Ltd., UK) used as the DNA size marker and lane 3 is the negative control with water blank instead of template DNA.

Serial Number - 1	Clone – 27-GV002
Category - RT-PCR	Size of the PCR band - 170bp
Primers - RF1 & RR2 nested with RF1 & RR3	Length of the sequence – 64 bases

Sequence

```
 1    GCCAGTGTCT GGTCCATGCA GAATACAAAC CCTTCTGGGC AAAGGCTCTT
51    TGTTCCCACA CATC
```

Homology

Hits	Sequence	Length	Extent
33	Drosophila melanogaster genomic scaffold 142000013385789	30,581	18/18
	Human Xp22 BAC GSHB-590J6	195,142	18/18
	Human DNA sequence from clone RP4-563E14 on chromosome 20	28,519	18/18

Serial Number - 2	Clone – 29-GV003
Category - RT-PCR	Size of the PCR band - 170bp
Primers - RF1 & RR2 nested with RF1 & RR3	Length of the sequence – 96 bases

Sequence

```
 1    TCCACAAAGG CCAGGATGAG CATATACAGC ATAGATCCAA TCTCTGCACT

51    GACTGCCCAC TGAAAATGGG CAAATGTGCA TGTCATCCCA GGTAAT
```

Homology

Hits	Sequence	Length	Extent
20	Human chromosome 4 clone B344K19 map 4q25	163,712	20/20
	Human DNA sequence from clone 125N5 on chromosome 6q26-27	84,126	19/19
		166,436	18/18
	Human clone RP11-506M9		

Table 3.3a: Sequences obtained from the 170bp RT-PCR bands amplified first with primers RF1 & RR2 and then nested with primers RF1 and RR3 from the chicken adipose tissue total RNA. The amplified bands were ligated with the plasmid pBSKII+, transformed into E.coli and sequenced using Thermosequenase sequencing kit. All the sequences deciphered by the manual sequencing procedure were analysed with the GCG programme of the Wisconsin package for the presence of the vector and any one of the primers used. Sequences after removing the vector and primer sequences were compared with the Genbank database by NCBI blast search through the world wide web. From the list of the homologous sequences, three sequences showing the highest degree of homology are shown in the table.

Serial Number - 3	Clone - 13-GN13
Category - RT-PCR	Size of the PCR band - 170bp
Primers - RF1 & RR2 nested with RF1 & RR3	Length of the sequence -106bases

Sequence

```
  1  TCGAGGTCGA CGGTATCGAT AACGTTGATG GCCAGTGTCT GGTCCATGAT
 51  AGAGTCAGTT ACTGGGTCGA CATAGCCAAT TGCCATTCAG TGGCATCATC
101  AAATGT
```

Homology

Hits	Sequence	Length (bp)	Extent
613,978	No significant matches or high noise		

Serial Number - 4	Clone - 29-GN29
Category - RT-PCR	Size of the PCR band - 170bp
Primers - RF1 & RR2 nested with RF1 & RR3	Length of the sequence -52bases

Sequence

```
  1  TCGAGGTCGA CGGTAACGAT AAGCTTGATC CCAAAAGGCC AGGATGACGA
 51  TA
```

Homology

Hits	Sequence	Length (bp)	Extent
613,978	No significant matches or high noise		

Table 3.3b: Sequences obtained from the 170bp RT-PCR bands amplified first with primers RF1 & RR2 and then nested with primers RF1 and RR3 from the chicken adipose tissue total RNA (continued). The amplified bands were ligated with the plasmid pBSKII+, transformed into E.coli and sequenced using Thermosequenase sequencing kit. All the sequences deciphered by the manual sequencing procedure were analysed with the GCG programme of the Wisconsin package for the presence of the vector and any one of the primers used. Sequences after removing the vector and primer sequences were compared with the Genbank database by NCBI blast search through the world wide web. No homologues could be found in the database for these sequences.

Serial Number - 5	Clone – 31-GN31
Category - RT-PCR	Size of the PCR band - 170bp
Primers - RF1 & RR2 nested with RF1 & RR3	Length of the sequence – 132 bases

<div align="center">Sequence</div>

```
  1    CAAAAAAGGA CAGCATGATG AGCAAATACA GGCGGAATAT CCAGGAGCGA
 51    GATTAGCTGC ACTGTCTCCT TTGTAGAGAT GTGGAGTGTG GAAGAGTCGA
101    GAGGCTGTGG GTGAGATGGG ATACATGACA GA
```

<div align="center">Homology</div>

Hits	Sequence	Length	Extent
6	Caenorhabditis elegans cosmid Y97E10B	36,785	18/18
	Mus musculus chromosome 17, clone 29_N_7	201,986	18/18
	Mus musculus major histocompatibility locus class III region: butyrophilin-like protein gene	201,964	18/18

Serial Number - 6	Clone – 35-GN35
Category - RT-PCR	Size of the PCR band - 170bp
Primers - RF1 & RR2 nested with RF1 & RR3	Length of the sequence – 107 bases

<div align="center">Sequence</div>

```
  1    CACAAAGGCC AGCATGACAT ACTGGGTTAT ACCAAATATA TCAGGGCAAC
 51    TTCTGTAGGC ATGGTCTGGT CCATTGTGTC ATCACTTTGG ACAAGAGCTT
101    GCCAGAG
```

<div align="center">Homology</div>

Hits	Sequence	Length	Extent
16	Human BAC clone CTB-57J11 from 7q21.1-q22	168,056	19/19
	Drosophila melanogaster genomic scaffold 142000013386052 section 2 of 5	296,279	18/18
	Mus musculus protein kinase C, lamda (Pkcl), mRNA	1,966	18/18

Table 3.3c: Sequences obtained from the 170bp RT-PCR bands amplified first with primers RF1 & RR2 and then nested with primers RF1 and RR3 from the chicken adipose tissue total RNA (continued). The amplified bands were ligated with the plasmid pBSKII+, transformed into E.coli and sequenced using Thermosequenase sequencing kit. All the sequences deciphered by the manual sequencing procedure were analysed with the GCG programme of the Wisconsin package for the presence of the vector and any one of the primers used. Sequences after removing the vector and primer sequences were compared with the Genbank database by NCBI blast search through the world wide web. From the list of the homologous sequences, three sequences showing the highest degree of homology are shown in the table.

Figure 3.5: The two PCR amplified bands from chicken genomic DNA with primers RF1 and RR2. There were two bands at 2Kb and 1.2Kb with primers RF1 and RR2, which were consistently amplified. These bands were cut out (shown along lane 1) and used for further nested PCR. Lanes 2-4 show bands from chicken genomic DNA amplified with other combinations of primers, which were produced with single primer. Lane 5 shows the negative control without gDNA and lane 7 is 100bp ladder DNA size marker. The Mg^{++} concentration was 3mM and the annealing was at 55°C throughout.

Figure 3.6: The PCR bands amplified with primers RF1 and RR2 nested with RR3 and a degenerate primer RF3. Both of the heavy (2Kb) and light (1.2Kb) PCR bands obtained from the gDNA with primers RF1 and RR2 with 3mM Mg^{++} and 55°C annealing (Figure 3.2a) were subsequently used for nested PCR with different combinations of primers. The 2Kb band did not yield any further bands while the 1.2Kb band yielded two new bands of ~950bp and ~850bp size (lane 3) after nesting with RR3 and a degenerate primer RF3. Upon cloning and sequencing, none of the sequences showed homology with any known genes or the presence of any of the primers used. Lane 1 shows the 100bp ladder plus (MBI Fermentas, Vilnius, Lithuania through Helena Biosciences Ltd., UK) used as the DNA size marker and lane 2 shows the bands obtained with semi-nesting with a single primer RR3.

from the amplification of the 1.2Kb band with nested primers. Further cloning and sequencing revealed that one of the sequences (850bp) was chicken apolipoprotein B (table 3.4a). The sequence of the other band (950bp) was not significantly homologous to any known genes in the database (table 3.4b). Comparison of the sequences with the Swissprot database with a blastx function of NCBI did not reveal any similarity with known proteins or any functional domains within the proteins.

3.3.3 Attempts to amplify the putative chicken leptin gene described by Taouis et al. (1998) and Ashwell et al. (1999a) from chicken nucleotide templates

RT-PCR amplification of DNA from (broiler and bantam) chicken liver and adipose tissue and mouse fat total RNA was performed using primers RF4 and RR4 (figure 3.2), which share complete identity with the mouse and the published chicken leptin sequences (Taouis *et al.* 1998; Ashwell *et al.* 1999a). A magnesium concentration of 2.5mM was used. After preheating the blocks to 94°C, the PCR cycling conditions were one cycle at 94°C for 4 min., 60°C for 1 min., and 72°C for 1 min and then 39 cycles of 94°C for 1 min., 60°C for 1 min. and 72°C for 1 min with the final extension cycle at 72°C for 5 min. As expected, mouse fat RNA generated a band of 400bp but no band of a similar size were generated from the chicken RNAs (figure 3.7a). The chicken RNA templates were subjected to identical PCR conditions as above using chicken glyceraldehyde phosphate dehydrogenase (GAPDH) primers (see table 3.1). A strong band of the predicted 345bp was obtained confirming the integrity of the chicken RNA samples and the efficiency of reverse-transcription process by the presence of amplification products consistently in all chicken lanes (result not shown). Southern hybridization with a mouse leptin cDNA probe using low stringency hybridization and washing conditions generated a conspicuous signal from band amplified from mouse RNA. There was no signal at all in any of the chicken lanes (figure 3.7b).

Serial No. Genomic 1	Clone(s): 33 and 43
Fragment size: ~850base pairs	Sequence length: 150 bases

Consensus with the contig fragment assembly

```
3        chlepb1_43   +------------->
  2      chlept1_33   +--------------->
  c      CONSENSUS    +--------------->
                      |----------|----------|----------|----------|----------|
                      0          100        200        300        400
```

Consensus sequence

```
1    GTGCAACTCT GTGGAGTACA yTAACCAAAT GCCACAGAAA AGCAGAGAGG CACTCAGAAA

61   TAAAGTGGAA CTAACACGCG CGCTAATTCA GCAAGGCGTT GAACAAGGCA CCAGGAAATG

121  GGAAGAAATG CAAGCATTCA TTGATGAACA
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

```
tmpseq_1 |
         0              50             100
```


Hits	Sequence	Length (bp)	Extent
	Chicken apoB mRNA encoding apolipoprotein	1,484	137/138
35	Arabidopsis thaliana chromosome II section 28 of 255	105,222	28/30
	Arabidopsis thaliana chromosome II section 85 of 255	81,298	28/30
	Genomic sequence for Arabidopsis thaliana BAC T22C5 from chromosome I	106,753	28/30

Table 3.4 a: The consensus sequence of the chicken genomic PCR. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained from the sequences first amplified from chicken genomic DNA with primers RF1 and RR2 (as in figure 3.5) and then the 1.2kb band nested with primers RF3 and RR3 (as in figure 3.6). A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambiguous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No. Genomic 2	Clone(s): 23, and 35, 37 and 45
Fragment size: ~950base pairs	Sequence length: 183 bases

Consensus with the contig fragment assembly

```
5      chlept1_37  +--------------->
   4      chlept1_35  +--------------->
   3      chlepm1_23  +----------------->
   2      chlepb1_45  +---------------->
   C      CONSENSUS   +----------------->
                      |----------|----------|----------|---------|---------|
                      0         100        200        300       400
```

Consensus sequence

```
  1   CCTCGCCAAG ACTATGGTGA GTAGTGAAGG CTCTCTTACA AAACCCAGAG TTTTCTCAGA

 61   AGTACCCCTT CACTAACCAG CTGAGCTGAA TGAACTGCAA GAGGTACTCA GAATTGGGAG

121   TAGGTTAGCC AGTCTGAGAG CCAACTCCAT TTGATGGCAG ATAGCCTGAA CATACATTGC

181   AGT
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

Hits	Sequence	Length (bp)	Extent
	Gallid herpesvirus 1 ICP18.5 gene	31,424	22/22
	Human BAC clone GS1-542D18 from 7q31-q32	141,120	19/19
19	Homo sapiens chromosome 17, clone hRPK.214_O_1	166,687	22/23
	Arabidopsis thaliana chromosome II section 44 of 255	94,503	18/18

Table 3.4 b: The consensus sequence of the chicken genomic PCR. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained from the sequences first amplified from chicken genomic DNA with primers RF1 and RR2 (as in figure 3.5) and then the 1.2kb band nested with primers RF3 and RR3 (as in figure 3.6). A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambiguous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Figure 3.7: a: RT-PCR of mRNAs from mouse abdominal fat (lane2), broiler chicken liver and fat (lanes 3 and 4) and bantam chicken liver and abdominal fat (lanes 5 and 6). Primers used were RF4 and RR4, sharing complete identity with both mouse and chicken sequences. **3.7: b**: Southern analysis of the gel in 'a', showing that hybridization of a mouse leptin cDNA probe of 348bp size as mentioned in section *4.3.8* (chapter 4) is confined to the amplification product from mouse fat mRNA
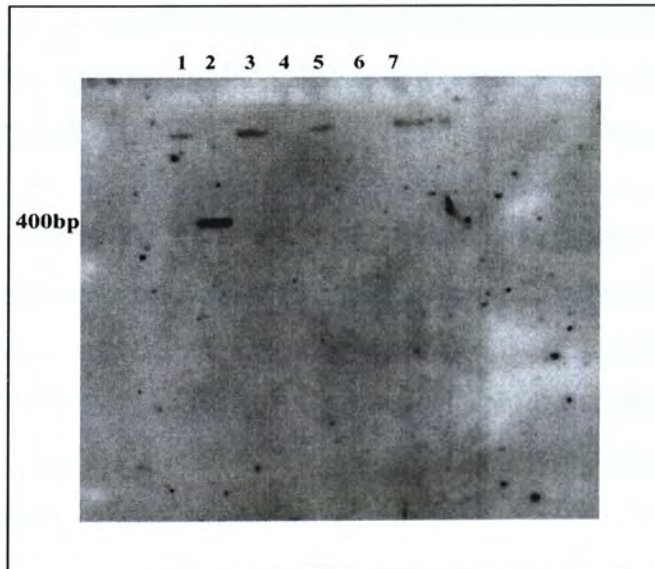
3.6 Discussion

The use of several RT-PCR reaction conditions, primers, and templates derived from fat and liver RNA and gDNA from different strains of chickens should have amplified the chicken leptin sequence if it shares close similarity with the mouse. The series of experiments described above failed to amplify a mouse-like leptin sequence from chicken templates using various combinations of the primers listed in table 3.1, all of which amplified the leptin sequence from mouse adipose tissue mRNA, but not mouse liver mRNA. The sequence information in these series of experiments reveal that what appeared as amplified bands from chicken cDNA and gDNA templates were highly heterogenous and completely unrelated to mammalian leptin sequences (tables 3.2, 3.3 and 3.4). Similar attempts to amplify the templates from chicken and other avian species also failed to demonstrate any homology between the avian and mammalian leptin sequences (Friedman-Einat *et al.* 1999; T. Okhubo, Centre for Molecular Biology & Genetics, Mie University, Tsu, Japan; G. Graham, Department of Animal Sciences, University of Western Australia; S. Takeuchi, Okayama University, Japan, personal communications).

The chicken leptin sequence reported by Taouis *et al.* (1998) was generated by RT-PCR from chicken fat and liver mRNAs using primers based on the mouse leptin sequence. The authors reported that the resulting PCR product shares 95% identity with mouse leptin at the nucleotide level. However, even when primers that completely matched both the published mouse and chicken leptin sequences were used, mouse-like leptin sequences were not obtained from any of the chicken templates whereas consistent amplification of leptin sequences was obtained from mouse adipose tissue mRNA. Other primer pairs homologous to the published sequence also failed to amplify predicted size bands from chicken templates. These results do not confirm sequence similar to that reported by Taouis *et al.* (1998) and Ashwell et al. (1999a) or a highly homologous gene to mammalian leptin in the chicken.

The failure to confirm the findings of Taouis *et al.* (1998) and Ashwell *et al.* (1999a) using RT-PCR could be due to low expression of the gene in the adipose tissue and liver used to prepare the cDNA templates for RT-PCR. Mammalian studies show that the major source of leptin is white adipose tissue and its expression is generally high in normal fully fed subjects and only decreases markedly after long-term food deprivation (Zhang *et al.* 1994; Trayhurn 1995a,b; Hardie *et al.* 1996b; Zheng *et al.* 1996). In the present experiment subcutaneous and visceral adipose tissue and liver were collected from birds known to be fully fed before sampling, and the tissues were snap frozen and stored at -70°C until the RNA was extracted. Thus, the possibility of low leptin gene expression in the tissues collected for heterologous RT-PCR cloning should have been avoided. The remote possibility of low leptin gene expression in adipose tissue and liver was circumvented by the use of genomic DNA as a template. The failure to amplify leptin sequence from this source could be a consequence of long stretches of introns in the genomic DNA. However, some of the primers used were entirely within the mammalian leptin exons and assuming the intron-exon structure was the same in the chicken, this should not have been a problem. The most obvious possibility is that there is a low homology between mammalian and avian leptin genes than expected or claimed by Taouis *et al.* (1998) and Ashwell *et al.* (1999a). This could prevent the amplification of both gDNA and cDNA because the primers contain too many mismatches. Logically this seems the most likely explanation for the failure of the attempt to clone the chicken leptin gene using RT-PCR.

CHAPTER 4

SOUTHERN ANALYSES OF CHICKEN LEPTIN -LIKE
SEQUENCES

4.1 Introduction

In the previous chapter, attempts to clone the chicken leptin gene by RT-PCR failed, and the published chicken leptin sequence (Taouis *et al.* 1998; Ashwell et al. 1999a) could not be confirmed using the primers and PCR conditions specified by the authors. With the failure of RT-PCR to clone the chicken leptin gene, it was essential to confirm the existence of a homologue by Southern hybridization, that is, Zhang *et al.*'s (1994) observation that there is a leptin-like sequence in the chicken genome. Southern hybridization would also provide a means of validating leptin DNA probes, which could then be used to probe cDNA and genomic DNA libraries (chapter 5).

The principle of hybridization analysis is that a single-stranded DNA or RNA molecule of defined sequence (the "probe") forms base-pairs bands with a second DNA or RNA molecule that contains complementary bases (the "target"). The stability of the resulting double stranded hybrid polynucleotide depends on the extent of matching of base pairs (Ausubel *et al.* 1998). The innovative idea of immobilizing hybridizing DNA/RNA on a solid support was first proposed by Denhardt (1966) and led to methods for identification of specific sequences in genomic DNA. The "Southern blotting and hybridization" technique combining agarose gel electrophoresis and nucleic acid reassociation was developed by E.M. Southern (1975). He devised a method to make it possible to transfer DNA/RNA molecules in an electrophoresis gel to a membrane (filter). Single-stranded DNA or RNA is thereby bound in such a way that it is prevented from reacting with itself and is available to form hybrids with single-stranded nucleic acid added in solution. In Southern blotting genomic DNA is digested with one or more restriction enzymes, and

the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured *in situ* and transferred from the gel to a filter. The relative positions of the DNA fragments in the electrophoresis gel are preserved during their transfer to the filter. The DNA attached to the filter is hybridized to a probe sequence in solution, which is labelled with reporter molecules (radiolabeled or fluorescent-labeled). After hybridization, excess probe is removed by washing and hybrids on the filter are visualized by the reporter molecules.

The aims of the present chapter were:

(1) to test the hypothesis that a leptin-like sequence exists in the chicken genome by Southern hybridization of mouse leptin probes with chicken genomic DNA;

(2) to predict the homology of the chicken leptin gene relative to mammalian sequences by comparing Southern hybridization of a mouse leptin probe with chicken, mouse and sheep gDNA under varying stringencies.

4.2 Methods

4.2.1 Extraction of genomic DNA

The genomic DNA (gDNA) was extracted from liver, lungs, brain and kidney of mouse (preserved frozen at -70°C) using a commercial kit (Nucleon ST kit, Amersham Life Science) according to the manufacturer's instructions. Sheep gDNA from embryonic liver was kindly provided by Dr. L. Young, Roslin Institute. Chicken gDNA was prepared as described in Chapter 2 (section 2.7). During the DNA extraction procedure, vortexing was avoided to minimize single-stranded nicks and double-stranded breaks. Precipitated genomic DNA was incubated overnight at 37°C to solubilize fully. The concentration of extracted DNA was calculated from the absorbance reading at 260 and 280nm. To assess the quality of the genomic DNA extracts, they were subjected to electrophoresis

overnight on 0.7% normal agarose gel in 1×TAE containing ethidium bromide at 25-30 volts. Samples showing signs of smearing or of RNA contamination were discarded. Genomic DNA stocks in water containing RNAse A, were stored in a refrigerator at 4°C up to 2 weeks to minimise the introduction of nicks through repeated freeze-and-thaw cycles. Samples for long-term storage were divided into several aliquots and frozen. Aliquots were thawed as needed and stored at 4°C.

4.2.2 Restriction enzyme digestion

For restriction enzyme digestion, EcoRI, HindIII and a combination of BamHI and HindIII were chosen as they were expected to yield uniformly cut small size fragments (Sambrook *et al.* 1989). About 30-40 µg gDNA giving 50% recovery after ethanol precipitation was digested with one or more restriction enzymes. Digestion was performed in a 1.5 ml Eppendorf tube with 4-5 units of EcoRI, HindIII or BamHI and HindIII/µg gDNA, together with the appropriate 10× buffer. The concentrations of DNA in preparations of high molecular weight mammalian gDNA were often so low that it was necessary to carry out restriction digestion in relatively large volumes (300-400µl). The chief problem encountered during digestion of high molecular weight DNA was unevenness of digestion caused by clumps of DNA. To ensure homogeneous dispersion, DNA was digested for several hours, usually overnight, in a shaking incubator and a further aliquot of restriction enzyme was added. The fragments of DNA were concentrated by precipitation with absolute ethanol, followed by washing in 70% ethanol and stored at 4°C. The samples were then briefly dried under vacuum before being reconstituted in water. The digested gDNA was quantified spectrophotometrically by noting the absorbance at 260 and 280nm. The reconstituted digested gDNA was stored at 4°C for short periods and at -20°C for longer periods.

4.2.3 Electrophoresis

The amount of genomic DNA needed to generate a detectable hybridization signal depends on a number of factors, including the proportion of the genome that is

complementary to the probe, the size of the probe and its specific activity, and the amount of genomic DNA transferred to the filter. Under optimal conditions, the method reportedly detects less than 0.1 pg of DNA complementary to a probe radiolabelled with ^{32}P to high specific activity of $>10^9$ cpm/µg (Sambrook *et al.* 1989). Thus, a sequence of 1000 base pairs (bp) that occurs only once in the genome (i.e., 1 part in 3 million) can be detected after an overnight exposure of 10 µg of genomic DNA transferred to the filter and hybridized to a probe several hundred nucleotides in length. Practically, the detection limit for a radioactive probe with a specific activity of 10^8 to 10^9 dpm/µg is about 0.5 pg DNA (Ausubel *et al.* 1995). Thus, for mouse genomic DNA, 10 µg - equivalent to 1.5 pg of a single-copy gene, 500 bp in length - is a reasonable minimum quantity to load. As the probe length was smaller (348 bp) in the present case, and because the homology between the mouse probe and the chicken leptin sequence was expected to be low, a larger amount was used as suggested by Birren *et al.* (1997). Usually 15 µg of digested chicken gDNA was loaded per well onto a thin (<7mm) 0.7% agarose gel. Signal quality did not improve when larger quantities were loaded. Ethidium bromide (0.5 µg/ml) was added to both the gel and the electrophoresis buffer. About 200 ng of low and high molecular weight marker DNA (100 bp ladder and Lambda DNA digested with Bsp68I (NruI) & XhoI respectively) was also loaded to enable visualization with ethidium bromide. Electrophoresis was performed in 1× TAE buffer under low voltage (<1 V/cm) to prevent smearing of the DNA fragments. Towards the end of each run, serial dilutions of pBSK$^+$II vector containing a 348 bp mouse leptin cDNA insert were loaded as a positive control. Plasmids were diluted in carrier solutions of chicken gDNA digested with EcoRI. Plasmid concentration was normally 0.1-10 × the predicted copy number of the gDNA. After electrophoresis, a digital image of the gel was captured using GelDoc imaging software (Bio-Rad Laboratories Ltd, Hertfordshire, UK.). A transparent ruler was placed alongside the gel to calculate the size of any hybridizing bands.

4.2.4 Preblotting preparation

For depurination, the gel block was rinsed in water before immersion in $10 \times$ gel volume of 0.25M HCl for 30 min. The gel was rinsed again in water before transfer to $10\times$ the gel volume of 0.4M NaOH for 20 min for complete denaturation of the genomic DNA. Finally, the gel was washed in water before proceeding with blotting.

4.2.5 Blotting by downward capillary transfer

Downward capillary transfer was performed as detailed in section 2.15.2.

4.2.6 Immobilization

After blotting, the membrane was rinsed in $2\times$ SSC, placed on a sheet of freshly cut Whatman 3MM filter paper and allowed to air dry as specified by the manufacturers. The membrane was stored wrapped in 3MM paper in a dry place at room temperature until further use.

4.2.7 Prehybridization

A variety of both aqueous and formamide-based hybridization solutions were tested, and optimal results were obtained with an aqueous solution described below. The same aqueous prehybridization (APH) stock solution was used for both prehybridization and hybridization. This consisted of 50mM sodium phosphate (pH 7.4), 1mM Na_2 EDTA, and 7% SDS in water. An APH working solution was made by the addition of sonicated salmon sperm DNA as a blocking agent to prevent non-specific background hybridization. Other blocking agents tested such as yeast tRNA and Denhardt's solution, were found to be less effective. Sonicated salmon sperm DNA (5mg/ml) was denatured by boiling at 95°C for 10 min and was immediately placed on ice for 2 min. The denatured salmon sperm DNA was then added to the APH stock solution to a final

concentration of 100µg/ml. Prehybridization and hybridization were normally carried out in a hybridization oven (Biometra Ltd, Maidstone, Kent, UK). Nylon membranes with immobilized gDNA were moistened in 6× SSC (to prevent bubble formation during prehybridization and hybridization) before transfer to a clean bottle containing working APH solution equilibrated at the prehybridization temperature at a volume of 1ml/10 cm^2 membrane. Membranes were then incubated in the hybridization oven with rotation for 2 h at 57°C.

4.2.8 Leptin and GAPDH probe preparation and ^{32}P labelling

The leptin probe used for Southern analysis was a 348-base pair fragment of mouse leptin cDNA corresponding to positions 134 to 481 of the coding region of the mRNA sequence (Zhang *et al.* 1994; accession number: U18812). Probe template was prepared by PCR amplification from a plasmid containing this sequence using primers RF2 and RR1 (see chapter 3 and figure 4.1).

A chicken glyceraldehyde 3-phoshate dehydrogenase (GAPDH) cDNA probe template (344bp long from nucleotide 900 to 1243 of chicken GAPDH mRNA, accession number - KO1458, Version: KO1458.1 GI:211800 of Genbank) was prepared similarly from the plasmid construct with the chicken GAPDH sequence insert by PCR amplification using the following primers:
Forward: GAPDHFor (19mer)- TGT GAC TTC AAT GGT GAC A
Reverse: GAPDHRev (19mer)- GCT GAT AGA AAC TGA TCT G

The PCR amplified products were run on a 3% agarose gel (figure 4.2) and the bands were cut out from the gel and purified using the Geneclean (Anachem Ltd., Vista, CA) kit. Template concentration was determined by absorbance at 260 nm on a spectrophotometer and the sequence was confirmed by Thermosequenase cycle sequencing as described in chapter 2.

```
SCORES              Init1:  1392 Initn:  1392 Opt:  1392
              100.0% identity in 348 bp overlap


                                          10        20        30
probe_                           AGTCCAGGATGACACCAAAACCCTCATCAA
                                 |||||||||||||||||||||||||||||||
mouse1 GTCTTATGTTCAAGCAGTGCCTATCCAGAAAGTCCAGGATGACACCAAAACCCTCATCAA
          110       120       130       140       150       160


              40        50        60        70        80        90
probe_ GACCATTGTCACCAGGATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mouse1 GACCATTGTCACCAGGATCAATGACATTTCACACACGCAGTCGGTATCCGCCAAGCAGAG
          170       180       190       200       210       220


              100       110       120       130       140       150
probe_ GGTCACTGGCTTGGACTTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mouse1 GGTCACTGGCTTGGACTTCATTCCTGGGCTTCACCCCATTCTGAGTTTGTCCAAGATGGA
          230       240       250       260       270       280


              160       170       180       190       200       210
probe_ CCAGACTCTGGCAGTCTATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mouse1 CCAGACTCTGGCAGTCTATCAACAGGTCCTCACCAGCCTGCCTTCCCAAAATGTGCTGCA
          290       300       310       320       330       340


              220       230       240       250       260       270
probe_ GATAGCCAATGACCTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAG
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mouse1 GATAGCCAATGACCTGGAGAATCTCCGAGACCTCCTCCATCTGCTGGCCTTCTCCAAGAG
          350       360       370       380       390       400


              280       290       300       310       320       330
probe_ CTGCTCCCTGCCTCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGA
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mouse1 CTGCTCCCTGCCTCAGACCAGTGGCCTGCAGAAGCCAGAGAGCCTGGATGGCGTCCTGGA
          410       420       430       440       450       460


              340
probe_ AGCCTCACTCTACTCCAC
       ||||||||||||||||||
mouse1 AGCCTCACTCTACTCCACAGAGGTGGTGGCTTTGAGCAGGCTGCAGGGCTCTCTGCAGGA
          470       480       490       500       510       520
```

Figure 4.1:   Position of the mouse cDNA probe used in Southern hybridisation: The 348bp sequence is shown to be 100% identical over a complete overlap, from nucleotide 134 to 481, in the coding region of the mouse leptin gene (Genebank, accession no. U18812) using Wisconsin GCG-8.1 programme.

95
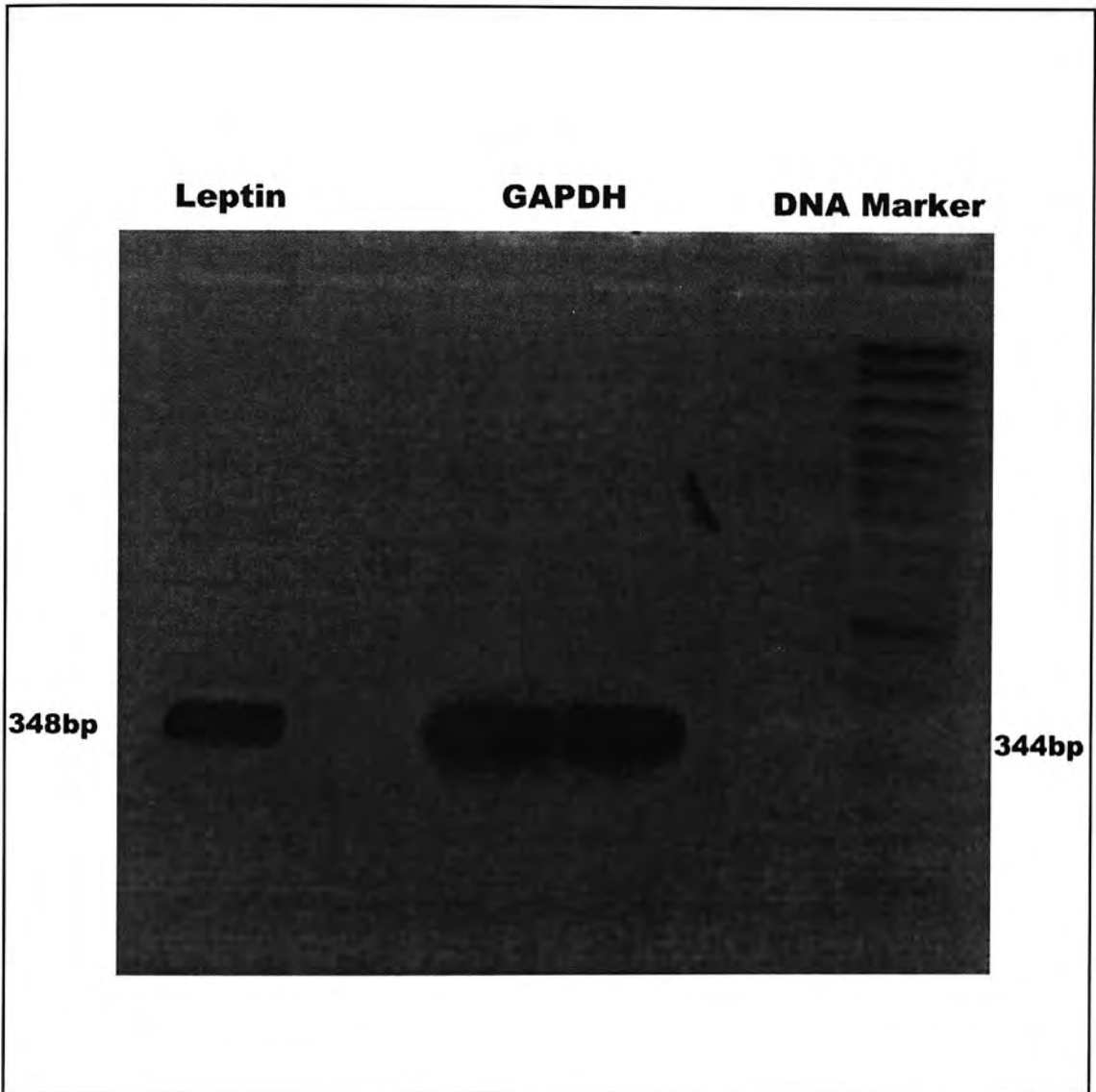
Figure 4.2: The PCR amplified probe templates run on a 3% agarose gel before cutting out the bands. RT-PCR of mouse fat RNA with primers RF2 and RR1 yielded a band size of 348bp. The GAPDHfor and GAPDHrev primers yielded a 344bp band from the PCR with plasmid constuct as the template. The bands were cut out and the DNAs were purified which were used as the template for leptin and GAPDH probes made by random primed method, respectively. The DNA size marker used is 100bp ladder plus.

The probe was labelled by random-priming based on the method of Feinberg and Vogelstein (1983, 1984). Two different methods were used:

4.2.8.1 ^{32}P labeling: method 1

Approximately 30 ng of template DNA in a volume of 33 µl in water was denatured at 95°C for 3 min and immediately placed on ice. The following reagents were then added to the template solution in order:-

10 µl of OL buffer (made up from a ratio of 100:250:150 of solution A, [1 ml of 1.25MTris-HCl, pH 8.0, 0.125M MgCl$_2$; 18 µl 2 β-mercaptoethanol; 5 µl of dATP; 5 µl of dTTP; 5 µl of dGTP], solution B [2M HEPES, titrated to pH 6.6 with 4M NaOH] and solution C [hexadeoxyribonucleotides evenly suspended at 90 OD 260U/ml], respectively);

1 µl of 10 mg/ml BSA;

5 µl of [α ^{32}P]-labelled dCTP (220 TBq/mmol, 37.0 MBq/100 µl) and

1 µl of Klenow enzyme (large fragment of DNA polymerase 1.5 U/µl).

All components were mixed together by gentle pipetting. The reaction tube was then incubated in a perspex box at room temperature for 4-5 h.

4.2.8.2 ^{32}P labeling: Method 2

A solution of template containing 22.5 ng DNA in 30 µl water was denatured at 95°C for 3 min and then immediately placed on ice for 2 min. The denatured template was then added to a solution containing 10 µl of 5× OLB, 1 µl of purified BSA, 1 µl of Klenow fragment and 5 µl of [α ^{32}P] dCTP. The total volume was made up to 50 µl with water and the tube was incubated at room temperature in a perspex box for more than 3 h.

For both methods, the probe was purified by phenol-chloroform extraction. Thirty µl of TE equilibrated phenol and 30 µl of chloroform were added to the labelled probe solution, and the tube was vortexed and microfuged. The tube was then placed in a dry

heating block at 70°C for 2 min, briefly vortexed and and heated again at 70°C for another 5 min before being vortexed and microfuged at 13,000 rpm for 4 min. A 50 μl aliquot of the supernatant aqueous phase was placed onto a TE Midi Select-D G-50 column (5 Prime 3 Prime Inc., CPLabs, Bishops Stortford, Herts, UK), microfuged, and the eluate was collected.

4.2.9 Scintillation counting of probe solution

A 1/200 dilution was prepared by pipetting out 0.5 μl of column eluate and mixing with 99.5 μl of water. Ten μl of the diluted probe solution was mixed with 3 ml of the Scintillation cocktail fluid (Fisher Chemicals, Loughborough Leicester, UK). The tubes were counted in a liquid scintillation counter (Wallac-1410, Wallac Oy, Turku 10, Finland) with the cocktail fluid as blank. Only probes with specific activities of $>1 \times 10^8$ d.p.m./μg were accepted for further use (for details of the calculation see appendix).

4.2.10 Hybridization procedure

An appropriate volume aqueous hybridization (AH) solution was made from APH stock solution by the addition of 10% w/v poly ethylene glycol equilibrated to the hybridization temperature. The addition of poly ethylene glycol increases the rate and efficiency of the nucleotide hybridization (Amasino 1986). The probe solution was denatured at 95°C for 3 min and placed on ice for 2 min. The denatured probe was then mixed into the equilibrated AH solution to a minimum concentration of 1×10^6 d.p.m./ml. On completion of the prehybridization reaction, the APH working solution was poured off and immediately replaced with the AH solution containing the denatured probe. Membranes were hybridised for 16-18 h at 57°C, to allow the formation of mismatch duplexes (see appendix). Diffusion of the probe into the filter (Anderson 1999) was ensured by using a small reaction volume and by shaking or rotating the reaction vessel.

4.2.11 Washing

Following hybridization, membranes were rinsed in 2× SSC and then taken through a series of 30 min. washes with increasing stringency. Low stringency washes began with 2× SSC; 0.1% SDS at 59°C. Higher stringency conditions were obtained by increasing the temperature of incubation and decreasing the salt concentration (by diluting the 2 × SSC further in water).

Radioactive hybrids bound to the membrane were then analyzed using phosphor imaging or autoradiography.

4.2.12 Phosphor imaging

Washed membranes generated in the hybridization experiments were wrapped in Saran wrap and mounted on absorbent paper marked with radioactive ink for orientation. Membranes were then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for various length of time. Digitised images were captured and visualized using the NIH Image program on an Apple Macintosh computer.

Radiation emitted by the radioactive hybrid hits the phosphor imaging screen creating a latent image (Johnston *et al.* 1990). The length of time required to obtain an image depends on the amount of radioactivity present. Compared with autoradiography phosphor imaging is 10-100 times more sensitive (Anderson 1999) and the linear range of detection is double. Screens were reused after bleaching by exposure to light.

4.2.13 Autoradiography

When there were a lot of hybridized nylon membranes to be examined autoradiography was used. Since the sensitivity of autoradiography is lower than the phosphor imager potentially weaker signals were detected by longer exposure. Autoradiographs were made

by exposing the membranes (wrapped in Saran wrap) to Kodak X-OMAT (XAR) films placed in X-OMATIC C2 cassettes with intensifying screens (Kodak Scientific Imaging System through Anachem, Luton, Beds, UK) for a period of 5-21 days. During this period the cassette was kept at -70°C. X-ray film was developed automatically using the Xograph Compact X2 film processor (Xograph Ltd, Malmesbury, Wiltshire, UK).

4.2.14 Control hybridization with a chicken GAPDH probe

In order to confirm the quality of the chicken gDNA and to demonstrate that the hybridization protocol was correctly carried out, a control chicken GAPDH probe was included. The mouse leptin probe was stripped off from the experimental membranes after hybridization, washing and autoradiography. This was done by washing the membrane in 0.1% SDS; 0.01× SSC at 99°C twice for 1h. The stripped membrane (wrapped in saran wrap) was loaded against a phosphorimager screen for 10 days. Then the screen was developed to confirm that the probe had been removed. The membrane was stored wrapped in Whatman filter paper at room temperature until further use.

After prehybridization at 55°C in APH solution with salmon sperm DNA as the heterogeneous blocking agent, the stripped membranes were transferred to AH solution containing 10% PEG and the random-primed chicken GAPDH cDNA probe. The membranes were incubated overnight in the hybridization oven in rotating glass tubes at 55°C. On completion of hybridization the membranes were rinsed in 0.1%SDS; 2 × SSC at room temperature for 5min. and then washed twice (30min. each) in 0.1%SDS; 0.2× SSC at 55°C. The washed membranes were exposed to a phoshorimager screen for 48 h before distinct signals were seen in the chicken gDNA lanes.

4.3 Results

4.3.1 Southern hybridization of the mouse leptin probe with chicken genomic DNA

The membranes hybridized under moderate stringency at incubation temperatures of 57°C (for the leptin probe) and 55°C (for the control GAPDH probe) displayed hybridization bands consistently at 21kb and 16.4kb along the mouse gDNA digested with HindIII, at 10.1kb along the sheep gDNA digested with EcoRI, at 1.4kb and 1kb along the sheep gDNA digested with HindIII and at 2.7kb and 2.5kb along the chicken gDNA digested with HindIII (figures 4.3 and 4.4).

After washing at moderate stringency (0.1% SDS; 0.4× SSC at 50°C) all the mouse, sheep and chicken gDNA lanes contained bands hybridising to the mouse leptin probe (figure 4.3). The mouse gDNA produced 21kb and 16.4kb bands after HindIII-digestion and a 6.4kb band after HindIII + BamHI-digestion. The sheep gDNA produced a faint 27.5kb band and a strong 10.1kb band after EcoRI digestion. Bands of 2.7 and 1.0kb were observed for sheep genomic DNA digested with HindIII, and 2.4 and 0.6kb bands for sheep genomic DNA digested with a combination of BamHI and HindIII. The chicken gDNA produced multiple bands when digested with EcoRI, HindIII and HindIII + BamHI (figure 4.3, table 4.1).

A single 2.5kb band was observed to hybridize to chicken gDNA digested with HindIII when the filter was washed at a higher stringency (0.1% SDS; 0.4× SSC at 59°C; figure 4.4). Under this washing condition, no band was observed to hybridize to chicken gDNA digested with EcoRI. A hybridization band of low molecular size of ~1.4kb was more prominent in the mouse gDNA digested with EcoRI. The intensity of the signals (figure 4.3 and 4.4) was mouse > sheep > chicken.

4.3.2 Southern hybridization of a mouse leptin probe with chicken, mouse and sheep gDNA at various stringencies

Southern hybridization membranes produced in experiment 1 showing clear bands were further washed using gradually increasing stringencies, by both increasing the washing temperature and reducing the salt concentrations of the washing solution. Hybridizing bands were observed in gDNA from chicken, mouse and sheep after washing at 0.1%
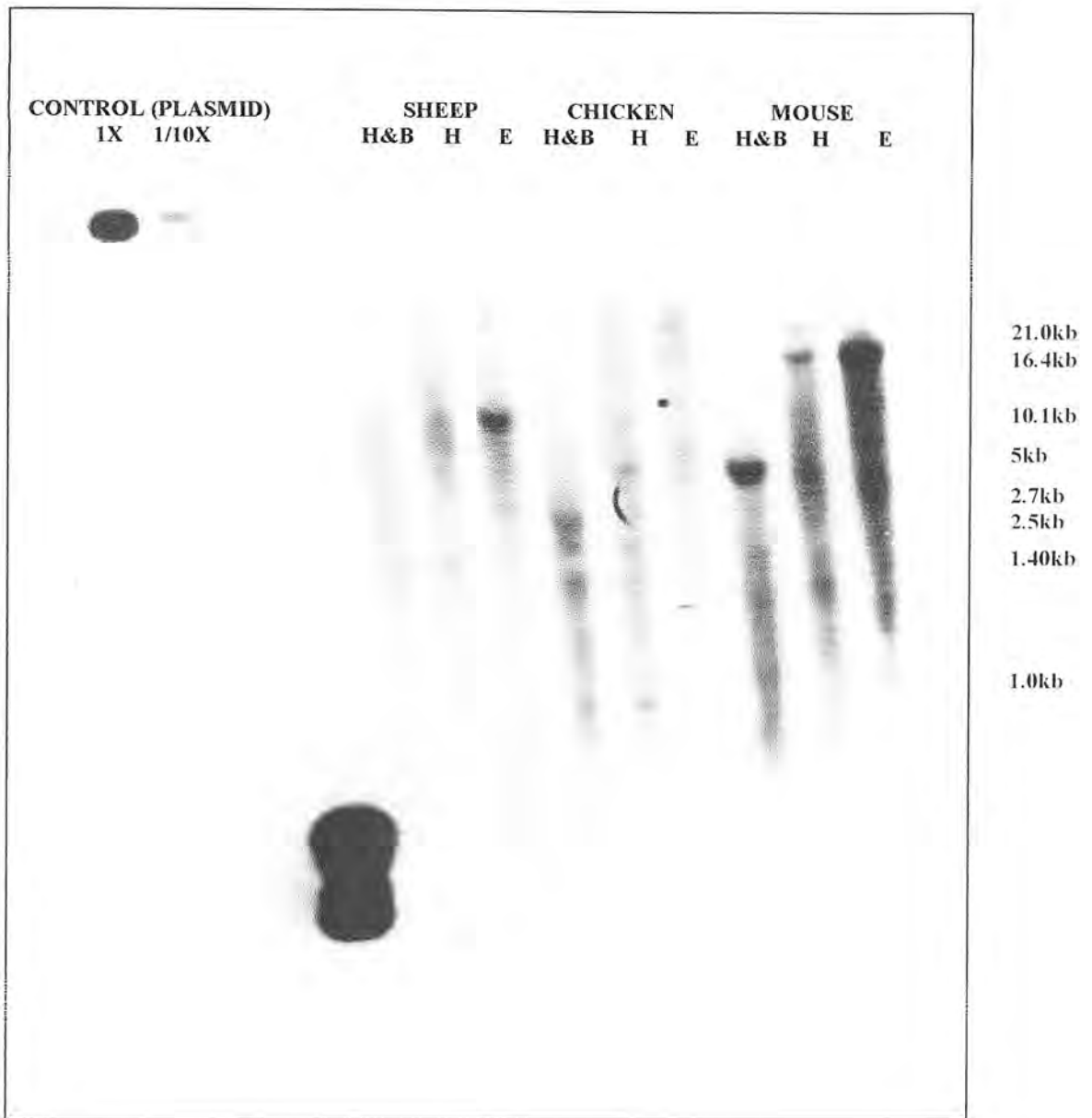
Fig. 4.3: Southern blots of mouse, sheep and chicken gDNA hybridized with mouse cDNA probe, washed in moderate stringency. Restriction-digested genomic DNA samples were Southern blotted, incubated with the mouse leptin probe of 348bp length. Washing was at moderate stringency (0.1% SDS; 0.4× SSC, 50°C -30min ×2) and exposure to Phosphor imager screen for 72h. As a positive control, the probe sequence inserted in plasmid construct were loaded ((1× and 1/10 × as much the expected copy number as in the test gDNA lanes) towards the end of electrophoresis. High and low molecular weight markers as described in chapter 2 were also loaded. E- EcoRI, H- HindIII and **B&H**- BamHI and HindIII, the restriction enzymes used to digest the respective gDNA samples.

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Sl No.	Band Name	MW (approx.)
1	EcoRI-1	20.8kb
2	EcoRI-2	11.3kb
3	EcoRI-3	5.5kb
4	EcoRI-4	1.9kb
5	HindIII-1	15.7kb
6	HindIII-2	10.1kb
7	HindIII-3	6.7kb
8	HindIII-4	2.4kb
9	HindIII-5	1.6kb
10	BamHI +HindIII-1	4.0kb
11	BamHI +HindIII-2	2.2kb
12	BamHI +HindIII-3	1.6kb

Table 4.1: Description of the multiple bands observed along the chicken gDNA. The membrane was washed in 0.1%SDS; 0.4×SSC at 50°C. These bands are shown in figure 4.3a.
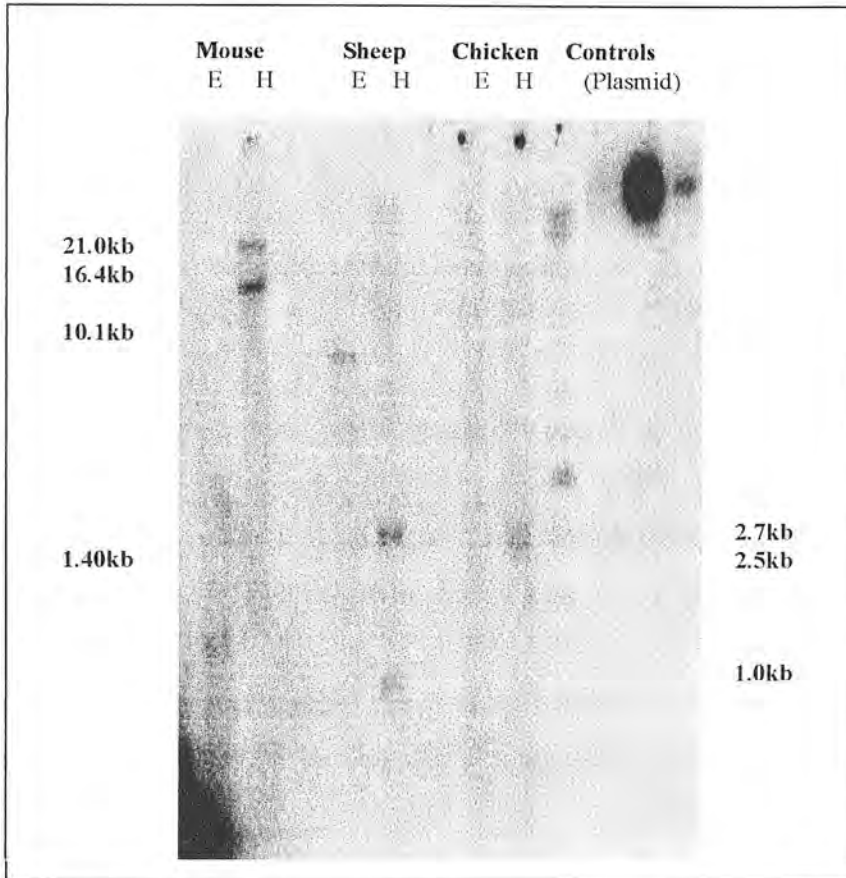
Fig. 4.4: Southern blots of Mouse, Sheep and Chicken gDNA hybridized with mouse cDNA probe, washed in moderate stringency. Restriction-digested genomic DNA samples were Southern blot hybridised with a mouse leptin probe. The membranes were washed in 0.1% SDS; 0.4× SSC, 59°C -30min. × 2, and exposed to Phosphor imager screen for 72h. As a positive control, the probe sequence inserted in plasmid construct were loaded ((1× and 1/10× as much the expected copy number as in the test gDNA lanes) towards the end of electrophoresis. High and low molecular weight markers as described in chapter 2 were also loaded. **E**- EcoRI, **H**- HindIII, the restriction enzymes used to digest the genomic DNA samples .

SDS; 0.4× SSC at 50°C and 59°C (low stringency) (Figure 4.3 and 4.4). However, after washing at 0.1% SDS; 0.2× SSC at 66°C (moderate stringency), the chicken bands disappeared while those of the sheep and mouse bands were retained (figure 4.5). The intensities of the hybridization bands were higher in mouse gDNA lanes than in the sheep gDNA lanes (figure 4.5).

At high stringency washing using 0.1% SDS; 0.1× SSC at 66°C all chicken and sheep bands disappeared whereas the homologous mouse band remained (figure 4.6). This observation was confirmed twice with different batches of the gDNA and with different membranes.

The pBSKII$^+$ construct containing the leptin probe sequence served as a positive control. The plasmid (with the 348bp insert in it) was serially diluted in sonicated carrier DNA and loaded 30 minutes before the end of 16-18h electrophoresis at concentrations of 0.1× and 1× the predicted copy number of the leptin gene in the loaded gDNA. The signals generated by the two concentrations of plasmid were strong and the difference in the intensity was consistent with the difference in copy number of the 348bp leptin sequence insert loaded (figures 4.3 and 4.4). This indicated that the technique was sensitive enough to detect the target gene.

Membranes were also blotted and hybridized with the chicken GAPDH cDNA probe using the same conditions as for the mouse leptin probe, except that the overnight incubation was at 55°C. When these membranes were washed in 0.1% SDS; 0.2× SSC at 55°C, chicken gDNA produced a strong hybridization band (result not shown).

4.4 Discussion

These experiments indicate that a leptin-like homologue is present in the chicken genome supporting the zooblot data presented by Zhang et al. (1994). However, under medium stringency washing, the chicken signals disappeared whereas sheep and mouse signals
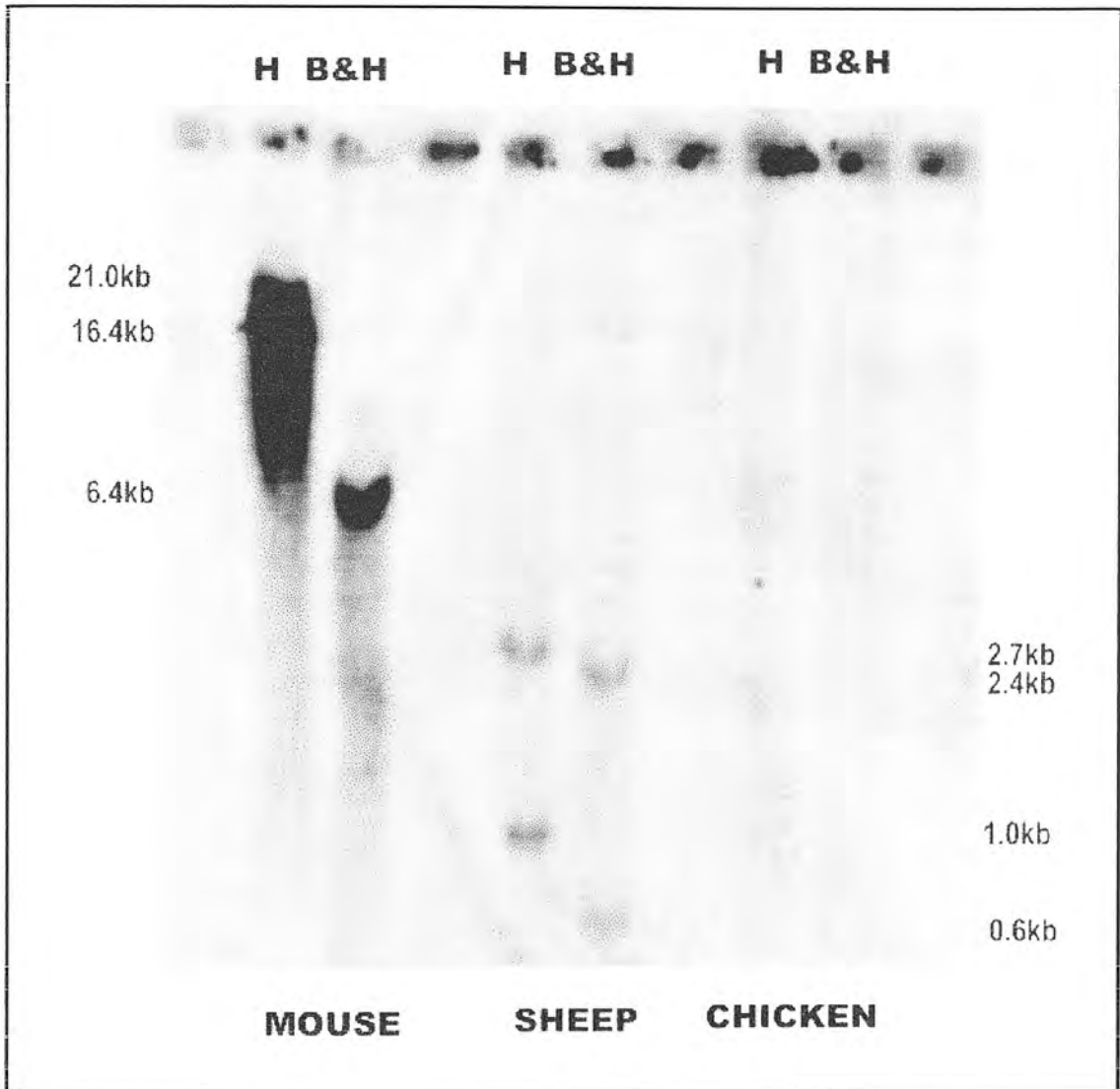
Fig. 4.5 Southern blots of Mouse, Sheep and Chicken gDNA hybridized with mouse cDNA probe, washed in moderately high stringency. Washing of the Southern blot membranes with the mouse, sheep and chicken genomic DNA was in 0.1% SDS; 0.2× SSC, 66°C -30min. % 2, and exposure to Phosphor imager screen for 72h. Mouse and sheep lanes show conspicuous signals whereas the signals along the chicken lanes have been removed. **H-** HindIII, **B&H-** BamHI and HindIII, the *restriction enzymes used to digest the genomic DNA samples.*
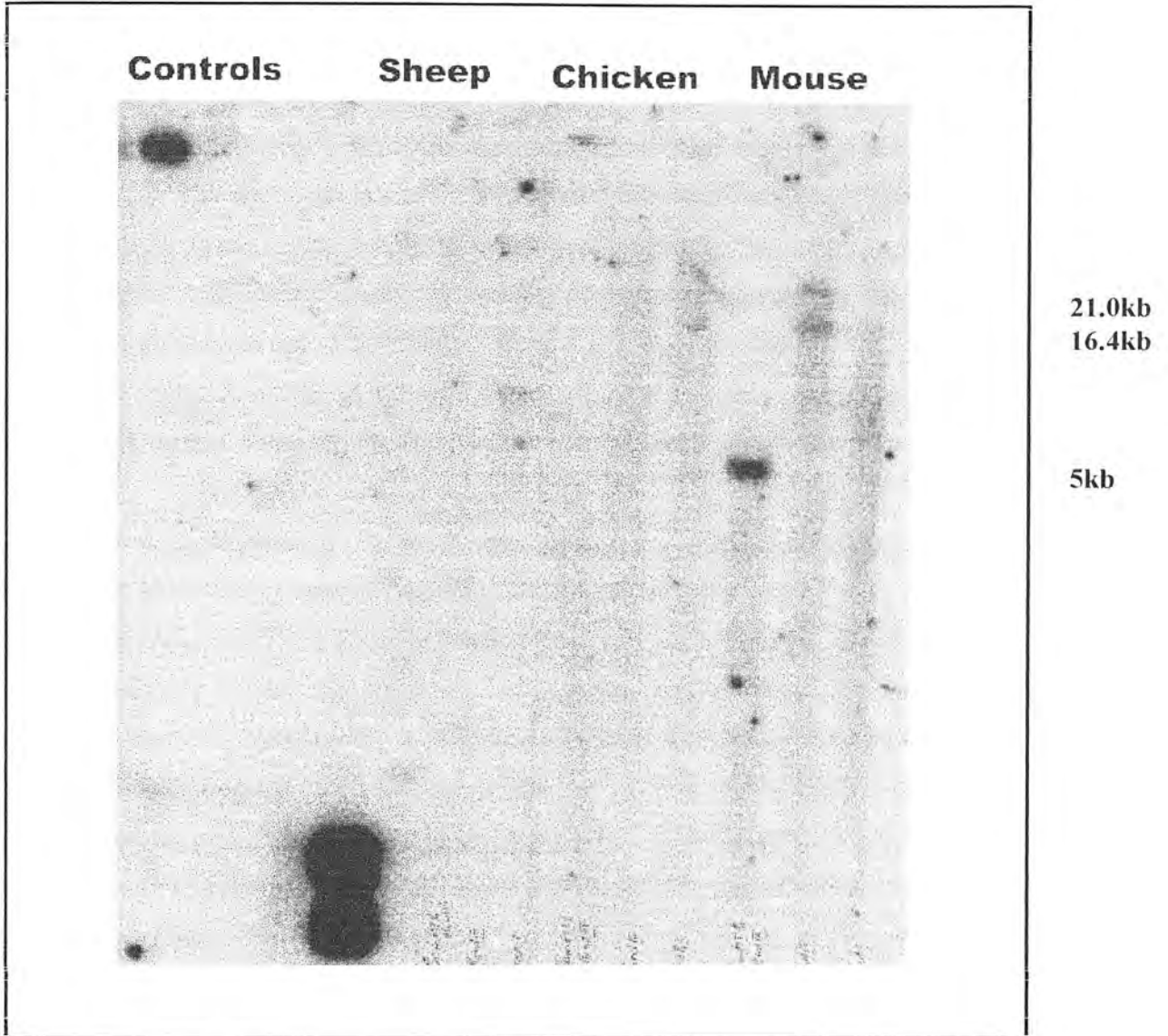
Fig. 4.6: Southern blots of Mouse, Sheep and Chicken g DNA hybridized with mouse cDNA probe, washed in high stringency. Washing of the membranes were in 0.1% SDS; 0.1× SSC - 66°C -30min × 2) and exposed to Phosphor imager screen for 10 days. As a positive control, the probe sequence inserted in plasmid construct were loaded ((1× and 1/10 × as much the copy number as in the test gDNA lanes) towards the end of electrophoresis. High and low molecular weight markers as described in chapter-2 were also loaded. The membrane is the same as in Figure 4.3a. The bands on the Mouse gDNA remain while those on the Sheep and Chicken gDNA lanes disappeared.

were retained. The homology of avian leptin-like sequence to the mammalian leptin sequence is therefore not equivalent. It is improbable that the putative chicken leptin sequence should be more than 83% homologous with the mouse sequence, since the sheep and mouse leptin nucleotide sequences are only 83% homologous. The mouse leptin probe covers 70% (348/504 bp) of the mammalian leptin coding region and shares 85.6% identity with the sheep leptin coding region. This assures a high probability that mouse leptin probe can distinguish differences in homologies between chicken, sheep and mouse gDNAs. Calculations based on washing stringencies adopted in this series of experiments (Meinkoth and Wahl 1984; Andersonon 1999; see appendix and table 4.2) indicate that sequence homology between chicken and mouse leptin gene is no more than 72 to 77% along the coding region. Of course, it could be much less than this.

An alternative explanation is that the hybridization signals obtained from the chicken gDNA lanes in this study were not specific for the chicken leptin gene. This is consistent with the disappearance of the multiple band pattern in the chicken gDNA lanes, observed at low stringency, when the stringency of washing was increased by raising the temperature by 9°C. Non-specific hybridisation of sequences other than leptin to the mouse probe might occur if the homology of chicken leptin to mouse leptin is lower than predicted above, or, if there is no leptin homologue in the chicken genome. These sequences may represent repetitive sequences which have low homology with the probe but are highly represented in the genome.

Regardless of whether the observed hybridisation results from low homology or non-specific hybridisation, the results are not consistent with the published chicken leptin sequences (Taouis et al. 1998; Ashwell et al. 1999a). Ashwell and co-workers (1999a) observed a single band on a chicken genomic Southern blot using a probe based on their published chicken leptin sequence. According to these authors, the pattern suggests the presence of a single leptin coding sequence in chicken genome. The general finding of a multiple banding pattern in the present study does not support this conclusion either. Although a discrete band was obtained on one membrane in the present study in a

Molarity [Na$^+$]	Tm (°C)	Washing Temp. (°C)	Tm-Washing Temp. (°C)	Observations
0.4	83	50	33	Mouse, sheep and chicken (multiple) bands visible.
0.4	83	59	24	Mouse, sheep and chicken bands visible.
0.2	78	66	12	Chicken bands have disappeared; sheep & mouse bands retained.
0.1	73	66	7	Only mouse bands visible.

Table-4.2: Comparison of Hybrid Stability in multi-species genomic Southern hybridization.

HindIII-digest (figure 4.4), the size was different to that observed by Ashwell *et al.* (1999a).

The present study does not support the existence of a mouse-like leptin sequence in the chicken genome. This view is reinforced by the fact that close similarity between the published chicken leptin sequence (Taouis *et al.* 1998; Ashwell *et al.* 1999) and mouse leptin is atypical of the phylogenetic relationships between leptin nucleotide sequences in other mammals, and between those of other cytokine genes in birds and mammals (see chapter 6). Overall, these results provide strong evidence that a mammalian-like leptin sequence is not present in the chicken genome. If a leptin gene is present in birds, it is likely that its sequence has considerably diverged from that of mammals.

CHAPTER 5

SCREENING OF CHICKEN RECOMBINANT DNA LIBRARIES
FOR LEPTIN-LIKE SEQUENCES

5.1 Introduction

The failure to amplify an homologous leptin sequence by PCR from chicken cDNA/gDNA (chapter 3) questioned whether a leptin-like sequence exists in the chicken genome. To test this, Southern blot analysis of restriction-digested mouse, sheep and chicken genomic DNA was carried out as described in chapter 4. Hybridization of chicken gDNA to a mouse leptin cDNA probe at low stringency suggested a homology of less than 72-77% between the two species. The experiments described in this chapter were therefore carried out to identify mouse leptin-like sequences in chicken cDNA/gDNA libraries.

A high-quality cDNA/gDNA library should contain all the sequences present in the original DNA mixture, and therefore contain at least one copy of the DNA sequence of interest. Hybridization to a specific nucleic acid sequence attached to a reporter molecule is the most commonly used screening method to select recombinant clones and was first developed for use with plasmids by Grunstein and Hogness (1975). This approach requires knowledge of the correct DNA sequence for use as a probe. If the DNA sequence of the desired DNA is not known, they can be deduced from a knowledge of the amino acid sequence of the protein of interest, even a partial amino acid sequence derived from microsequencing can be sufficient to allow eventual isolation of the entire gene (Gonzales-Villasenor and Manak 1998). Hybridization approaches, however, are not limited to screening with specific probes where the sequence is well characterized, since even very short stretches of sequence can be used to prepare oligonucleotide primers to generate the desired probe sequence following amplification by PCR of DNA.

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An appropriate cDNA library, constructed from mRNA expressing a target gene to be cloned in a tissue, is the first choice of recombinant DNA libraries for screening (Jerpseth *et al.* 1992). In contrast to the complexity of genomic DNA libraries, cDNA libraries represent only a small subset of genes likely to include the gene of interest. These libraries do not contain long stretches of noncoding regions or the untranslated intronic sequences, which typically break up individual genes and constitute the vast majority of DNA in a genome. The number of clones that must be screened in cDNA libraries is likely to be substantially less than the number that must be screened in gDNA libraries. The actual frequency of a given sequence in a cDNA library is determined by the abundance of the mRNA in the particular tissue or cell from which the library was prepared and can be estimated from the relative amount of the desired protein that is found in that cell or tissue. The level of expression is therefore a limiting factor in finding a target sequence in a cDNA library. Thus cDNA libraries are the first choice for heterologous cloning of genes.

Genomic DNA libraries are designed to represent the coding and noncoding sequences of the total genomic DNA for the donor organism, and are used for the isolation of complete gene clusters and retain the genetic organization and structure of gene including the regulatory and splicing signals. Though complex, genomic libraries avoid the risk of low levels of expression of the target gene to be cloned, inherent in cDNA libraries. An abundance of highly repetitive short stretches of DNA sequences in gDNA can cause very high background, which diminishes the sensitivity of the screening procedure (Gonzales-Villasenor and Manak 1998). The amount of work and the chances of success of identifying the target sequence by screening a gDNA library depend also on the vector system used.

Although plasmids are the simplest cloning vectors, their usefulness is limited by the very small size inserts they can hold, and therefore they are not used for constructing genomic DNA libraries. Early gene cloning experiments demonstrated the utility of bacteriophage lambda as a cloning vehicle was demonstrated (Murray and Murray 1974; Rambach and

Tiollais 1974; Thomas *et al.* 1974). The rapid deployment of lambda as a cloning vector was made possible by the wealth of detailed information already available on the organization and functioning of the lambda bacteriophage genome. The efficiency of preparing recombinant clones is generally greater in lambda than in plasmids (Dame 1998) and unamplified libraries are easily stored for long periods at 4°C due to the stability of the lambda phage particle. Lambda replacement vectors carry large inserts (up to 24kb) enabling easy screening, and are not subject to recombination (Frackman and Feiss 1998).

Identification of individual genes or gene clusters that span DNA fragments larger than 30 kb requires the selection of genomic libraries generated in specialized vector systems which can accommodate even larger inserts than the lambda phage vector. Vector systems such as cosmids, yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), and the P1 bacteriophage are useful in this respect since they can accommodate large chromosomal fragments. Cosmids undergo very little rearrangement upon propagation. Cosmid vectors are modified plasmids that can replicate DNA fragments 35 to 45 kb in length (Collins and Hohn 1978; Williams and Blattner 1980). An insert size of 35 to 45 kb in a cloning vector represents a feature that has particular advantages. They have a significant capacity, thus reducing the number of steps required to clone an entire gene or region, combined with very simple methods for isolating inserted DNA in pure form (Buxton *et al.* 1992). Cosmid vector minimizes the amount of screening to produce overlapping clones, which cover a particular region of the genome. This has been demonstrated in the isolation and mapping of large gene clusters or families like those of the ovalbumin (Royal *et al.* 1979), interferon-γ (Gross *et al.* 1981a,b; Lund *et al.* 1983, 1984), immunoglobulin (Cattaneo *et al.* 1981), histocompatibility complex (Steinmetz *et al.* 1982) and the T-cell antigen receptors (Griesser *et al.* 1988; Koop and Hood 1994). Cosmids, thus, have advantages of a plasmid vector combined with the large cloning capacity and efficient packaging system of lambda. Large genomic libraries may be prepared using this vector, which require for completeness only about half of the number of clones required for a lambda library.

The aim of this chapter was to obtain a chicken leptin homologue by screening the following libraries with a mouse leptin cDNA probe: a chicken adipose tissue cDNA library in lambda zap vector; a chicken genomic library in a lambda phage vector; and a chicken gDNA library in cosmid vector.

5.2 Methods

5.2.1 Screening of a broiler chicken adipose tissue cDNA library in lambda zap phage vector (Stratagene)

A cDNA library in lambda zap vector generated from adipose tissue of a 7-week old male broiler chicken, which had already been amplified once by the manufacturer was obtained from Stratagene. The cloning site was EcoRI and the recommended host was the BB4 strain of *E.coli*. The average insert size was estimated to be 1.0 kb, the primary plaque titre 2×10^6 plaque forming units/ml (p.f.u./ml) and the background of non-recombinants 6%. The estimated titre after one step of amplification is 1.1×10^{11} p.f.u./ml.

The colony lift procedure originally described by Grunstein and Hogness (1975) with modifications suitable for the phage lambda ZAP (Jerpseth *et al.* 1992) was used to screen the library. Libraries were grown out on agar plates to allow the formation of discrete plaques. Replicas of the plaques were made by overlaying the plaques with positively charged nylon membrane (Hybond XL, Amersham) and lifting the plaques. The DNA attached to the nylon membrane was denatured *in situ* with alkali and was immobilized onto the filter by baking. The filters were then screened by hybridization to mouse leptin cDNA probe under the conditions described in chapter 4. The position of positive signals could be keyed back to the original plate to isolate the plaques of interest using alignment marks.

5.2.1.1 Preparation of the cDNA library in BB4 E. coli

The *E.coli* BB4 strain was maintained on LB tetracycline agar plates. L-Broth containing 10mM MgSO4 and 0.2% maltose (50ml) was inoculated with a single bacterial colony and incubated overnight at 30°C in a shaking incubator. Forty milliliters of the culture was then poured into a 50ml polypropylene centrifuge tube and centrifuged at 2000 rpm for 10min. The supernatant was discarded and the BB4 cell pellet was suspended in 10mM MgSO4, aseptically. With frequent checking of the optical density at 600nm, the suspension was diluted further with 10mM MgSO4 until the final optical density reached 0.520, as recommended by the suppliers. A similar suspension, without maltose, was prepared for plating out the cDNA library.

An aliquot of the lambda zap containing the cDNA library was thawed and diluted in SM buffer in serial dilutions of 1/100, 1/1000, 1/10,000, 1/100,000 and 1/1000,000.

NZY bottom agar was melted in a microwave oven with frequent shaking and left to cool to about 55°C (hand hot). Approximately 80 ml was poured into 140 mm diameter sterile disposable plastic petri dishes to make a layer of about 5 mm thickness. Once set, these petri dishes were inverted and allowed to dry for about 20 min and were stored at 4°C, wrapped in sterile polythene, and used within 3 days.

The serial dilutions of lambda phage containing the cDNA library were added (3μl) to tubes containing BB4 bacterial suspension (600 μl). The tubes containing the BB4-phage mix were left at room temperature (~20°C) for 20 min. and were further incubated at 37°C for another 20 min. NZY top agar was melted in a microwave oven with frequent shaking. The molten NZY top agar was then poured into sterile 20 ml glass tubes, ~6.5 ml in each tube, and placed in a water bath at 48°C for about 20 min. NZY bottom agar plates (IPTG 0.5M, 50 μl /tube; X-GAL in dimethyl formamide 155 μl, 250mg/ml) dried in a 55°C oven for ~25 min was added to the tubes and vortexed before returning to the 48°C water bath. The BB4-phage mixes was then pipetted into the molten NZY top agar tubes containing IPTG and XGAL at 48°C. After quickly vortexing, the molten top agar was poured in to top of the dried NZY bottom agar plates. The top agar was spread

evenly over the bottom agar by rotating the petri dishes and allowed to set at room temperature. After about 20 min. the plates were placed in a 37°C oven and incubated overnight in an inverted position. The next day all the plated petri dishes were transferred to a 4°C room for about 2 h before counting the plaques.

The average percentage of white plaques (recombinant plaques) was 95% and the titre of library was 1.5×10^{11} p.f.u./ml. These observations matched Stratagene's description of the library.

5.2.1.2 Plating out the library

The library was plated out according to the manufacturer's instructions (see the calculation of the dilution factor in the appendix). The working phage solution, which was a 1/30,000 dilution of the stock library, was prepared by diluting a 1/300 diluted working stock a further 100 times. Ten microlitres of this diluted phage was used per 140 mm petri dish. However, ~700 µl of the BB4 suspension instead of the recommended 600 µl was used to slow down the plaque formation. All other plating steps were the same as described in section 5.2.1.1, except that IPTG and XGAL were not added in the NZY top agar. The plated library was incubated for 6-7 h to avoid the phage plaques merging to one another. The plates were incubated at 4°C for about 3 h before lifting the plaques.

5.2.1.3 Lifting the phage plaques formed by the plated out cDNA library and transferring them to nylon membranes

Twenty five positively charged, 132 mm diameter, nylon membranes (Hybond XL, version RPNS L/98/01, Amersham) were numbered with a pencil from 1 to 25 and a further 25 membranes were numbered from 26 to 50 to be used as replicates. Plaques were lifted from 5 agar plates at a time.

Nylon XL membranes 1-25 were placed gently on top of the NZY top agar plates with the cDNA library phage plaques with the numbered side facing up, using flat tipped forceps. The nylon membranes and the agar was pierced at four asymmetric edges for orientation with a sharp hypodermic needle dipped in Indian ink. After about 2 min., the membranes were gently lifted from the agar plates with flat tipped forceps to the denaturing solution.

Three plastic trays were prepared containing approximately 250ml of denaturing solution (1.5M NaCl; 0.5M NaOH) in the first tray; about 250ml of neutralizing solution (1.5M NaCl; 0.5M Tris-HCl, pH 8.0) in the second, and 100ml of 2 × SSC; 0.2M Tris-HCl, pH 7.5, in the third. The lifted nylon membranes were rinsed to get rid off any agar sticking to them and immersed in the denaturing solution for 2min. After denaturing, the membranes were transferred to the neutralizing solution in the second plastic tray for 5min. The neutralized membranes were then placed in 2 × SSC; 0.2M Tris-HCl, pH 7.5, in the third plastic tray for 30sec. The membranes were then blotted on Whattman 3MM blotting paper for about 10min. All the membranes were placed on blotting paper without overlapping and kept in an oven at 80°C for 2 h in order to immobilize the DNA. After immobilization the membranes were on blotting paper and stored at room temperature until used. The procedure was repeated for the duplicate replicate membranes, 26 to 50, except that these membranes were applied to phage plaques on the agar plates for 4 min instead of 2 min.

5.2.1.4 Prehybridization, hybridization and autoradiography of the nylon membranes

The nylon membranes with the immobilized DNA were wetted in 6 × SSC and prehybridized in aqueous prehybridization solution (7%SDS; 1mM Na_2EDTA; 50mM Sodium phosphate pH 7.4; sonicated salmon sperm DNA 100µg/ml) at 60°C for 3h with shaking at 90 cycles/min. After prehybridization the membranes were transferred to the hybridization solution (7%SDS; 1mM Na_2EDTA; 50mM Sodium phosphate pH 7.4; 10% PEG; containing the mouse leptin cDNA probe (100 µl/50 ml) denatured - at 95°C for 3

min.. The membranes were incubated overnight in the hybridization solution at 60°C with shaking at 90 cycles/min.

All the membranes were then washed twice in the washing solution (0.1% SDS; 0.2 × SSC) at 66°C for 30 min. each. During washing the total volume of the washing solution was 40 times of the hybridization solution, giving a total volume of 2 litres. The membranes were then transferred to autoradiograph cassettes for a period of 5-21 days. The autoradiograph plates were developed to visualize the hybridization of the mouse leptin probe to the cDNA library plaques.

5.2.1.5 Controls

The positive control for the screening procedure was a Hybond N$^+$ membrane blotted with pBSK II $^+$ plasmid containing the mouse leptin probe sequence. The negative control was a Hybond N$^+$ membrane blotted with pBSK II $^+$ plasmid containing a mouse NPY gene sequence. These membranes were processed in the same manner as the library membranes.

5.2.2 Screening of a chicken genomic library in lambda phage vector

A chicken genomic library in lambda phage vector EMBL 3 was kindly provided by Dr. Helen Sang, Roslin Institute. The host for the library was NM621 strain of *E. coli*.

The library was screened by nucleic acid hybridization using the colony lift procedure described for screening the adipose tissue cDNA library (section 5.2.1). Serial dilutions of the library were plated out and the titre determined. An appropriate number of plaques (usually 800-1000 plaques per petridish) were generated by plating out the appropriate dilution of the library. The plaque DNA was then immobilized onto positively charged nylon membranes (Hybond-N$^+$, Amersham), and hybridized to the mouse leptin cDNA probe as described for the adipose tissue cDNA library (section 5.2.1). Autoradiographs

were then prepared of the membranes as described previously (section 5.2.1.4) and used to identify plaques showing hybridization with the mouse leptin probe.

5.2.2.1 Identification of positive plaques

The developed autoradiographs were placed over the relevant nylon membranes on an illuminated viewing box to identify hybridizing plaques. The positions of the asymmetric Indian ink marks in the membranes were marked on the x-ray sheet with a marker pen so that the autoradiograph and the membranes were in correct orientation. The respective petri dishes were placed over the radiographic marks in the correct orientation with the help of the asymmetric marks on both the x-ray film and the petri dish. The positive plaques corresponding to the hybridization signals in the autoradiographs were marked with marker pen on the back of the petri dish.

5.2.2.2 Confirmation that positive plaques containing gDNA hybridizing to the mouse leptin probe

The positive plaques were picked using the tip of a sterile, flamed, glass pastette pipette attached to a rubber teat, pierced through the agar surface to the appropriate position of a petri dish. After rotating the pipette, the entire column of the agar with the plaque on the surface, was aspired and transferred to 1 ml of phage buffer in an Eppendorf tube. A drop of chloroform was added in order to prevent bacterial contamination. The tubes were stored at 4°C.

5.2.2.3 Secondary and tertiary screening of the library

In order to confirm that some plaques hybridize to the leptin probe it was necessary to undertake a further round of plating, hybridization and autoradiography.

To replate plaques in a secondary screening of the library, 10 µl of the phage sample stored were placed in a fresh sterile Eppendorf tubes and kept open in a 37°C water bath

for 1h to allow the chloroform to evaporate. Sterile phage buffer (300 µl) was added to the tubes aseptically to dilute the phage suspensions followed by vortexing and a centrifugation. From this diluted phage suspension, 3 µl was mixed with 100 µl of NM621 bacterial suspension in 10mM MgSO4 in a sterile glass tube. The phage-bacterial mix was left at room temperature for 15 min. and then placed in a water bath at 37°C for about 30 min. Further 1/10 dilutions were made as appropriate. The phage-infected cells were then plated as previously described (section5.2.1.2) and subjected to autoradiography.

Tertiary screening of the library was carried out on positive plaques of interest identified in the secondary screening.

5.2.3 Screening of a chicken genomic library in cosmid vector

A gridded chicken cosmid library from an adult Rhode Island Red female chicken white and red blood cells using supercos 1 as the vector and DH5α strain of *E.Coli* as the host was generously provided by Drs. Schalkwyk, Fries, Weiher and Buitkamp (University of Munich, Freising and Max Plank Institute of Molecular Genetics, Berlin, Germany). About 110,000 cosmid clones were grown and replicated in 384-well plates. An average insert size of 39kb was calculated from the analysis of 68 randomly selected clones with a range 20-48kb. A four-fold coverage of the genome is represented in the library as estimated from the insert length and number of recombinant clones (Buitkamp *et al.* 1998).

5.2.3.1 Primary screening

Primary screening of the cosmid library was carried out by J. Smith (Roslin Institute). The 384-well plates containing the replicated cosmid clones were immobilized onto a positively charged nylon membrane (Hybond-N+, Amersham) (each in duplicate). These were hybridized to the mouse leptin cDNA probe used for screening of cDNA and gDNA libraries (section 5.2.1.4). The hybridization solution was 10% PEG8000; 7% SDS; 1.5 × SSC. The filters were hybridized overnight at 50°C and then washed them at a stringency

of 0.2 × SSC; 0.1% SDS for 20min at 50°C. Where strong hybridization signals were observed the corresponding clones were obtained from Max Plank Institute of Molecular Genetics, Berlin, Germany, for further screening.

5.2.3.2 Secondary and tertiary screening

For secondary screening purified recombinant cosmid DNA from the primary screening was extracted using a Qiagen miniprep kit. After spectrophotometric quantification the recombinant cosmid DNA was cut with endonuclease PstI and the digested mixture was run out on a 1% agarose gel containing ethidium bromide. The gel was run for sufficient time to allow separation of the bands and an image of the gel under UV light was captured electronically. The electrophoretically separated DNA was depurinated with 0.25 M HCl, blotted onto a positively charged nylon membrane (Hybond-N$^+$, Amersham) with 0.4 M NaOH as the transfer buffer and immobilized by air-drying for 30min. These Southern blot membranes were hybridized with the mouse leptin cDNA probe as described in chapter-4. The positions of hybridization signals at moderate stringency were noted and the sizes of the fragments were calculated as described by Schaffer (1983). PstI-digestion and electrophoresis were repeated and the bands corresponding to the hybridization signals in the earlier step were cut out. By referring to the Southern blots, the regions of the gel containing the hybridization signals were cut out to subclone the DNA fragments they contained. In some cases four or five bands were cut separately when they were in the vicinity of a positive band.

In tertiary screening the subcloned plasmid DNA was extracted by the TENS miniprep method detailed in section 2.11 and the insert DNA was released by BamHI + HindIII digestion. Part of the digestion mixture was run on a 2% agarose gel containing ethidium bromide and subsequently subjected to Southern analysis using the mouse leptin cDNA probe used in library screening. Those subclones generating hybridization signals were further extracted, purified and sequenced. The tertiary screening thus covered all the subcloned DNA fragments.

5.2.3.3 Subcloning and sequencing of the DNA fragments

The DNA from the bands obtained in the secondary screening were ligated in to Blue script plasmid and transformed in *E. coli* DH5α as described in chapter 2 (section 2.19).

The positive subclones identified in the tertiary screening were extracted, purified and sequenced by Li Cor automatic sequencer as described in chapter 2 (section 2.20.3).

5.2.3.4 Sequence analysis of cloned DNA fragments from the tertiary screening

The Fragment Assembly System (FAS) of the Wisconsin UWGCG packages (Deverux *et al.* 1984) was employed to assemble the overlapping fragment sequences. The system makes it possible to: (1) store fragment sequences; (2) recognize overlapping sequences and create aligned assemblies, called contigs; and (3) display and edit the contigs.

The sequence data for a sequencing project were maintained and manipulated in a sequencing project database. Using the six programs of the FAS mentioned below, a separate project database for the entire sequencing project was created and maintained. The project database was created with GelStart, the fragment sequences entered with GelEnter, contigs were assembled with GelMerge, and assembled contigs were edited with GelAssemble. A schematic display of each of the consensus formed from the respective contigs was created with GelView.

The consensus directory has a consensus sequence file for each contig in the project database. Each contig is named after the left-most fragment in the alignment. Newly entered fragments and other unassembled fragments are also considered contigs and have a consensus sequence in the consensus directory. Because they do not yet align with any other contig, they are called contigs-of-one or single-fragment contigs. These latter contigs are not shown in the Results.

Comparison of all these consensus sequences were made using BLAST search in the National Centre for Biological Information (NCBI) site (http://www.ncbi.nlm.nih.gov/blast/).

5.3 Results

5.3.1 Screening of a chicken adipose tissue cDNA library

The chicken adipose tissue library was screened using new reagents and a mouse leptin probe, which did not yield any hybridization signals. The control pBSK II$^+$ plasmid, containing a mouse leptin sequence insert, included in the screening procedure generated a strong signal.

5.3.2 Screening of a chicken genomic library in a lambda vector

The first out of the two rounds of screening of the chicken genomic DNA library in lambda phage, yielded 19 positive signals. When these plaques were picked and plated out and screened again, 8 positive signals were found in five of the plates. After third phase screening four signals were found in two plates. However, these signals were weak, and after a further screening there were no positive signals in any of the petri dishes plated from these plaques. The experiment was repeated with another set of nylon membranes. During the first phase of screening there were 60 positive signals and all the 60 plaques were picked and plated out for secondary screening. In the secondary phase screening nine plaques appeared to be positive from five of the 60 plates. The nine plaques were picked and plated out to be screened further. There were no strong positive signals after the tertiary stage of screening. Thus, two separate screenings of the chicken genomic library in lambda phage vector did not yield a hybridizing plaque to be cloned and sequenced. The activity of the mouse leptin probe was confirmed by demonstrating that it hybridizes to the mouse leptin sequence cloned to a pBSK II$^+$ plasmid.

5.3.3 Screening of a chicken genomic library in a cosmid vector

Primary screening of chicken genomic library in cosmid vector with mouse leptin cDNA probe identified ten positive hybridization signals. When the cosmid DNA from these clones was extracted, digested and electrophoresed on agarose, seven out of the ten cosmids showed hybridization with the mouse probe. DNA from the corresponding regions of the agarose gel were cut out. In some cases four to five bands were cut separately corresponding to a single signal. Thus, there were 20 subclones from seven gel lanes with hybridization signal. When these subcloned DNA fragments were extracted, restriction-digested, run on agarose gels and subjected to Southern analyses, many of the plasmids hybridized to the mouse leptin probe. A typical example is shown (figure 5.1).

All the subclones were sequenced, which yielded 180 sequences: the poorly deciphered sequences and those with only the vector sequences were discarded. The remaining 145 sequences, with the vector sequence component removed, were entered into the fragment assembly system of the UWGCG programme (table 5.1a-o). When analysed in the fragment assembly system of GCG programme, there were 25 consensus sequences. When compared for homology with the published sequences in the database, some of them showed homology with known sequences (e.g. chicken Maf F gene, see table 5.2a-y). No sequence was found with homology to the mammalian leptin gene. However, there were short stretches within many of these sequences showing moderate homology with the mouse leptin probe used (see table 5.1a-o). Comparison of the sequences with the Swissprot database with a blastx function of NCBI did not reveal any similarity with known proteins or any functional domains within the proteins.

5.4 Discussion

Three chicken DNA libraries were screened for a mammalian-like leptin homologue using a mouse leptin probe. In two of the libraries, a chicken adipose tissue cDNA library, and a chicken gDNA library in lambda vector, no hybridization signals were identified. The third library, a chicken gDNA cosmid library, yielded a number of strong

Agarose gel electrophoresis
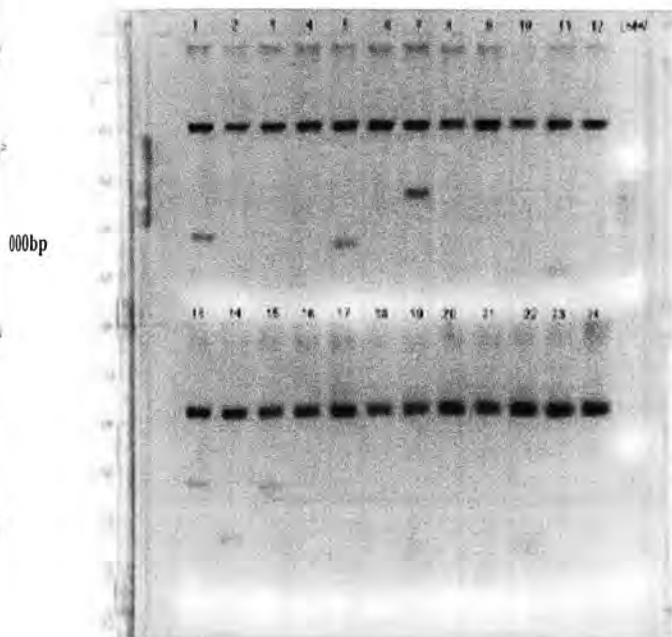
Southern blot hybridization

000bp

1000bp
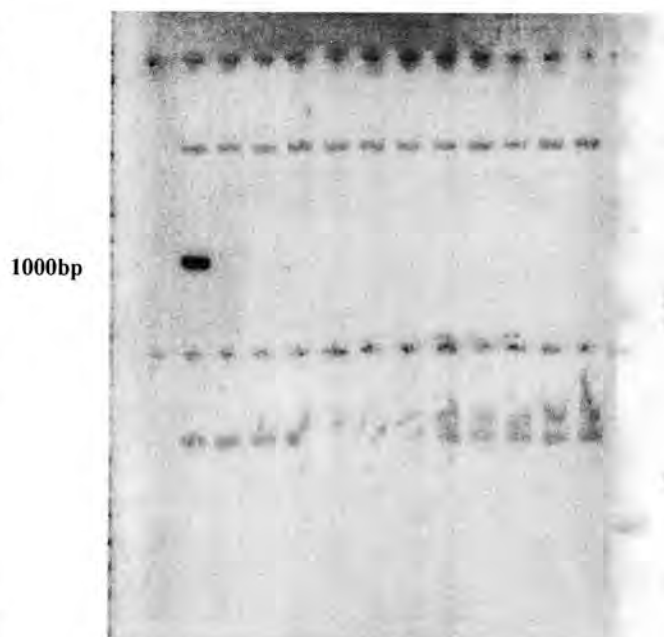
Figure 5.1: Tertiary screening of the chicken cosmid library: The cosmid clones hybridizing with the mouse leptin probe were identified in the primary phase of screening. The DNA extracted from these positive clones were further screened with the same probe in the secondary screening, which yielded 20 positive clones. The DNA bands corresponding to the strong hybridization signals from the secondary screening were cut out from the gel and ligated to pBSK II+ plasmid vector. During the tertiary screening, the insert DNAs were released from the plasmid vector by restriction enzyme digestion with HindIII and BamHI. **(a):** The digested plasmid with the inserts were electrophosed on agarose gel with ethidium bromide. The high molecular weight plasmid is visible in all the 24 gel lanes whereas the low molecular weight insert is present in only a few of the lanes. **(b):** The electrophoretically separated DNA were Southern blotted into positively charged nylon membranes, and then hybridized with the mouse leptin probe at moderate stringency. A positive hybridization signal is present only on lane 1. The sublones showing a positive hybridizing signal in the tertiary phase of screening were subsequently sequenced.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
1	1-1	150	fwd1,1,15,a	237	negligible	negligible	13	1. Human Chromosome 22q12 PAC Clone p704f1059q13 2. Human Chromosome 22q12 Cosmid Clone p42h1	173,029 45,436	26/28 26/28
2	1-1	150	rev1,1,15,b	378	negligible	negligible	-	No match found	-	-
3	1-1	150	rev1,1,5,b *	336	negligible	negligible	11	1. Drosophila melanogaster genomic scaffold 14200001386047 section 20 of 52 2. Drosophila melanogaster genomic scaffold 14200001386035 section 78 of 105	258,873 226,342	16/16 16/16
4	1-1	150	rev1,2,1,a *	150	negligible	negligible	22	1. Human 3q25-26 BAC CTB-177N7 2. Human BAC clone RP11-507E21 from Y	82,263 107,402	19/19 19/19
5	1-1	150	rev1,2,15,b	144	negligible	negligible	8	1. Sus scrofa thyroxine-binding globulin mRNA 2. Bos taurus thyroxine-binding globulin mRNA	1,284 1,280	16/16 16/16
6	1-1	150	rev1,2,3,a	140	negligible	negligible	7	1. Human DNA from chromosome 19-cosmid R28051 containing APLP1 2. Human 12 BAC RP11-575G13	39,146 157,673	21/21 20/20
7	1-1	150	rev1,2,3,b	410	negligible	negligible	51	1. Human chromosome 14 clone RP11-463C8 containing POMT2 gene 2. Human chromosome 14 clone RP11-493G17 and CTD-2516D11 map 14q24.3	180,331 210,204	19/19 19/19
8	1-1	150	rev1,2,7,a *	120	negligible	negligible	25	1. Human DNA sequence from clone 55I10 on chromosome 1q32.2-41 2. Human 3q25-26 BAC CTB-177N7	90,429 82,263	20/20 19/19
9	1-1	150	rev1,2,7,b	435	negligible	negligible	32	1. Streptomyces coelicolor cosmid 51A 2. Gallus gallus homeodomain-containing protein (GHox1i) mRNA	42,527 2,212	19/19 18/18
10	1-1	150	rev1,2,9,b *	130	negligible	negligible	35	1. Human 3q25-26 BAC CTB-177N7 2. Human BAC clone RP11-507E21 from Y	82,263 107,402	19/19 19/19

Table 5.1a: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

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| No. | Clone | Band size (bp) | File | Length (bp) | Similarity with probe | | Hits | Homology with known sequences | | |
					Percentage	Overlap		Sequence	Length	Extent
11	2-1	900	2_1_10	325	negligible	negligible	28	1. BAC R-417P24 of library RPCI-11 from chromosome 14 of Human. 2. Caenorhabditis elegans cosmid R07C3	169,235 40,254	20/20 20/20
12	2-1	900	2_11 *	651	60.3	78	43	1. Pheasant ovotransferrin(conalbumin) gene frag. repetitive seq 2. Chicken alpha-A-crystallin gene	739 6,311	40/44 33/35
13	2-1	900	2_1_14 *	289	66.7	42	31	1. BAC R-986E7 of RPCI-11 library from chromosome 14 of Human. 2. Human DNA sequence from PAC 333H9 on chromosome Xq26.1-q26.3.	214,527 42,098	23/24 20/20
14	2-1	900	2_1_23 *	237	64.0	50	21	1. Schizosaccharomyces pombe gene for Rfc3 2. Human chromosome 14 BAC containing gene for type 2 iodothyronine deiodinase (DIO2) gene	4,969 154,796	37/41 20/20
15	2-1	900	fwd2,1,10,c *	335	Negligible	Negligible	30	1. BAC R-417P24 of library RPCI-11 from chromosome 14 of Human. 2. Caenorhabditis elegans cosmid R07C3	169,235 40,254	20/20 20/20
16	2-1	900	fwd2,1,14,c *	255	66.7	42	31	1. BAC R-986E7 of RPCI-11 library from chromosome 14 of Human. 2. Human DNA sequence from PAC 333H9 on chromosome Xq26.1-q26.3.	214,527 42,098	23/24 20/20
17	2-1	900	fwd2,1,23,c *	437	91.7	24	98	1. Human chromosome 14 BAC containing gene for type 2 iodothyronine deiodinase 2. BAC R-959A22 of RPCI-11 library from chromosome 14 of Human.	154,796 168,677	20/20 20/20
18	2-1	900	fwdorrev2,1,14 *	402	64.7	51	-	No significant similarity found	-	-
19	2-1	900	rev2,1,14,a *	362	77.8	36	17	1. Human chromosome 16, cosmid clone RT99 (LANL) 2. Drosophila melanogaster genomic scaffold 14200001386035 section 66 of 105	40,419 233,313	21/22 19/19
20	2-1	900	rev2,1,17,d *	539	69.7	66	29	1. Human chromosome 14 BAC containing gene for type 2 iodothyronine deiodinase gene. 2. BAC R-959A22 of RPCI-11 library from chromosome 14 of Human.	154,796 168,677	20/20 20/20

Table 5.1b: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
21	2-2	900	2_2_5_*	885	60.5	81	42	1. Pheasant ovotransferrin(conalbumin) gene frag. repetitive seq 2. Chicken alpha-A-crystallin gene	739 6,311	40/44 33/35
22	2-2	900	2_2_8_*	657	negligible	negligible	26	1. Caenorhabditis elegans cosmid R10E4 2. Homo sapiens wingless-type MMTV integration site family, member 5A mRNA	36,170 4,114	22/22 21/21
23	2-2	900	rev2,2,15,a*	681	58.6	133	39	1. Chicken alpha-A-crystallin gene 2. Meleagris gallopavo clone TUCA930 microsatellite sequence	6,311 314	33/35 32/35
24	3-1	1000	3_1_6_*	460	91.7	24	16	1. Mus musculus leptin mRNA 2. Meleagris gallopavo leptin precursor mRNA	504 438	20/20 20/20
25	3-1	1000	rev3,1,1,a*	420	91.7	24	21	1. Aedes aegypti asparagine synthetase mRNA 2. Mus musculus leptin mRNA	2,169 504	20/20 20/20
26	3-2	1000	3_2_7_*	577	92.9	28	18	1. Human clone RP5-1193P9 2. Human DNA sequence from clone 468K18 on chromosome 6q24.1-24.3	133,991 137,387	21/21 21/21
27	3-2	1000	rev3,2,14,a*	744	56.8	88	24	1. Human clone RP5-1193P9 2. Human DNA sequence from clone RP4-742C19 on chromosome 22	133,991 122,748	21/21 21/21
28	3-2	1000	rev3,2,14,d*	470	72.4	29	23	1. Caenorhabditis elegans cosmid C27C12 2. Human clone RP11-440P12	36,047 171,881	22/23 20/20
29	3-3	1000	3_3_8_*	744	56.8	88	42	1. Gallus domesticus microsatellite marker MCW158 2. Gallus gallus microsatellite DNA (clone 63C12)	212 190	56/61 53/61
30	3-3	1000	rev3,3,6,a*	758	negligible	negligible	78	1. Gallus domesticus DNA microsatellite marker MCW158 2. Gallus gallus microsatellite DNA (clone 63C12)	212 190	56/61 53/61

Table 5.1c: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
31	3-4	1000	3_4_19 *	322	negligible	negligible	61	1. Human PAC clone RP4-715F13 from 7q11.21-q11.23 2. Human clone DJ1015P16A	145,878 174,725	21/21 21/21
32	3-4	1000	fwd3,4,19,c *	457	negligible	negligible	64	1. Human PAC clone RP4-715F13 from 7q11.21-q11.23 2. Human clone DJ1015P16A	145,878 174,725	21/21 21/21
33	5-1	500	fwd5,1,5,b *	515	91.7	24	75	1. Mus musculus leptin mRNA 2. Meleagris gallopavo leptin precursor mRNA	504 438	20/20 20/20
34	5-1	500	fwd5,1,6,a *	473	negligible	negligible	5	1. Human DNA sequence from PAC 179D3, between markers DXS6791 and DXS8038 on chromosome X 2. Human PAC clone RP4-740D2 from 7p14-p15	76,094 141,990	20/20 19/19
35	5-1	500	fwd5,1,7,a *	373	negligible	negligible	27	1. Drosophila melanogaster genomic scaffold 142000013386050 section 36 of 54 2. BAC R-543C4 of library RPCI-11 from chromosome 14 of Human	300,477 166,180	22/23 19/19
36	5-1	500	rev5,1,5,a	490	negligible	negligible	9	1. Human mRNA; cDNA DKFZp434K2235 (from clone DKFZp434K2235) 2. Human DNA sequence from PAC 358H7 on chromosome X	2,139 148,883	26/27 22/22
37	5-1	500	rev5,1,5,b *	435	55.3	47	14	1. Human DNA sequence from clone 579N16 on chromosome 22 2. Human arylsulfatase B (ARSB) mRNA	66,618 2,779	17/17 16/16
38	5-1	500	rev5,1,6,a *	504	63.3	49	24	1. Human BAC clone CTA-364P16 from 7q31 2. Human BAC clone CTB-191D16	97,943 174,952	20/20 20/20
39	5-1	500	rev5,1,7,a *	439	negligible	negligible	30	1. Oryza sativa genomic DNA, chromosome 6, clone:P0538C01 2. Arabidopsis thaliana DNA chromosome 4, contig fragment No. 57	155,634 199,577	23/23 21/21
40	5-1	500	rev5,1,15,a *	489	negligible	negligible	22	1. Dasypus novemcinctus mitochondrion 2. S.pombe chromosome II cosmid c28F2	17,056 39,320	23/24 20/20

Table 5.1d: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
41	5-2	500	fwd5,2,3,b	515	negligible	negligible	21	1. Human clone RP11-334E15 2. Human chromosome 19, cosmid F17972	183,666 42,244	20/20 19/19
42	5-2	500	fwd5,2,5,a *	535	negligible	negligible	8	1. Drosophila melanogaster genomic scaffold 142000013386055 section 9 of 63 2. Arabidopsis thaliana chromosome I BAC F24J13 genomic sequence	267,518 87,400	20/20 20/20
43	5-2	500	fwd5,2,8,a	408	negligible	negligible	57	1. Human chromosome 19, cosmid F16912 2. Drosophila melanogaster genomic scaffold 142000013386055 section 42 of 63	35,822 275,242	27/29 22/23
44	5-2	500	rev5,2,3,a	463	negligible	negligible	22	1. Caenorhabditis elegans cosmid W05B2 2. Oryza sativa genomic DNA, chromosome 2, clone:P0437H03	35,530 126,038	27/29 30/33
45	5-2	500	rev5,2,3,b	460	negligible	negligible	22	1. Caenorhabditis elegans cosmid W05B2 2. Oryza sativa genomic DNA, chromosome 2, clone:P0437H03 (contig b)	35,530 126,038	27/29 30/33
46	5-2	500	rev5,2,5,a	290	negligible	negligible	87	1. Human clone RP11-36B6 from 7q31 2. Human chromosome 7 common fragile site	192,768 161,155	22/22 22/22
47	7-1	800	7_30 *	588	negligible	negligible	19	1. Human DNA sequence from clone 431P23 on chromosome 6q27 (Contains the first coding exon of the MLLT4 gene for myeloid/ lymphoid or mixed-lineage leukemia) 2. Human genomic DNA, chromosome 6q27	147,971 331,211	23/23 23/23
48	7-1	800	7_31 *	509	negligible	negligible	16	1. Drosophila melanogaster genomic scaffold 142000013386035 section 105 of 105 2. Human 14q32 Jagged2 gene	249,284 148,083	19/19 19/19
49	7-1	800	7_33 *	638	54.6	141	69	1. Sus scrofa mRNA for soluble angiotesin-binding protein 2. Porcine DNA for endopeptidase 24.16, exon 12	3,819 226	22/22 22/22
50	7-1	800	7_34 *	420	67.3	55	15	1. E.caballus p53 gene exons 5 to 9 2. Equus asinus tumor suppressor (p53) gene, exons 5-9	1,339 1,380	20/20 20/20

Table 5.1e: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

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| No. | Clone | Band size (bp) | File | Length (bp) | Similarity with probe | | Hits | Homology with known sequences | | |
					Percentage	Overlap		Sequence	Length	Extent
51	7-1	800	7_35 *	379	80.0	25	40	1. Human BAC clone RP11-505D17 from 7p22-p21 2. Human DNA sequence from clone RP1-90L6 on chromosome 22q11.21-11.23	178,361 190,837	20/20 20/20
52	7-1	800	7_36 *	598	77.3	22	39	1. Human chromosome 14 clone RP11-463C8 containing POMT2 gene 2. Human target of myb1 (chicken) homolog (TOM1), mRNA	180,331 2,310	194/230 22/22
53	7-1	800	7_37 *	646	negligible	negligible	12	1. Human chromosome 14 clone RP11-463C8 containing POMT2 gene 2. Human PAC RPCI4-613B23	180,331 185,161	61/71 23/24
54	7-1	800	fwd7,1,23,a	716	69.2	39	49	1. Human chromosome 5 clone CTB-130E24 2. Mus musculus neural precursor cell expressed, developmentally down-regulated gene 5 (Nedd5), mRNA	126,169 3,170	20/20 20/20
55	7-1	800	rev7,1,10,a *	349	negligible	negligible	39	1. Drosophila melanogaster genomic scaffold 142000013385713 2. Human BAC clone GS1-113D4 from 5p15.2	67,870 154,028	20/20 20/20
56	7-1	800	rev7,1,23,a *	616	negligible	negligible	15	1. Human chromosome 17, Neurofibromatosis 1 locus 2. Mus musculus chromosome 10, clone RP21-247L16	297,898 130,427	22/22 23/24
57	7-2	800	fwd7,2,16,a	522	57.1	70	3	1. Homo sapiens hypothetical protein (HSU79253), mRNA 2. Homo sapiens clone 24431 mRNA	1,203 1,204	19/19 19/19
58	7-2	800	fwd7,2,5,a	604	negligible	negligible	46	1. Oryza sativa subsp. japonica BAC clone 34K24 2. Oryza sativa BAC clone 1.H19	142,852 77,605	22/22 22/22
59	7-2	800	fwd7,2,6,a *	671	negligible	negligible	88	1. Oryza sativa subsp. japonica BAC clone 34K24 2. Oryza sativa BAC clone 1.H19	142,852 77,605	44/44 44/44
60	7-2	800	fwd7,2,7,a *	629	66.7	45	32	1. Oryza sativa subsp. japonica BAC clone 34K24 2. Oryza sativa BAC clone 1.H19	142,852 77,605	22/22 22/22

Table 5.1f: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
61	7-2	800	fwd7,2,9,a	369	negligible	negligible	42	1. Strongylocentrotus purpuratus egg receptor for sperm mRNA 2. Human chromosome 17, clone hRPC.906_A_24	3,753 113,252	26/27 21/21
62	7-2	800	rev7,2,16,a *	358	negligible	negligible	3	1. Human hypothetical protein (HSU79253) mRNA 2. Human clone 24431 mRNA	1,203 1,204	19/19 19/19
63	7-2	800	rev7,2,5,a	342	negligible	negligible	38	1. Candida albicans strain SC5314 MTLalpha2, OBPalpha, PIKalpha, MTLalpha1, and PAPalpha genes 2. Candida albicans PIK1 gene	12,105 4,300	21/21 21/21
64	7-2	800	rev7,2,6,a *	345	negligible	negligible	74	1. Human clone RP11-255L1 2. Human Xp22 PAC RPCI1-5G11	162,608 92,975	26/28 20/20
65	7-2	800	rev7,2,7,a *	250	negligible	negligible	16	1. Human chromosome 16 BAC clone CIT987SK-723D3 2. Drosophila melanogaster genomic scaffold 142000013386043 section 6 of 8	49,746 274,591	44/44 42/42
66	7-2	800	rev7,2,9,a *	927	69.7	33	22	1. Arabidopsis thaliana DNA chromosome 3, BAC clone T12C14 2. Drosophila melanogaster genomic scaffold 142000013386047 section 37 of 52	77,720 261,694	24/24 22/22
67	7-4	800	fwd7,4,1,a	800	64.4	45	18	1. Human BAC clone CTA-257F18 from 7q31.1-q31.2 2. Drosophila melanogaster genomic scaffold 142000013386050 section 47 of 54	21,462 285,495	21/21 20/20
68	7-4	800	fwd7,4,15,a	780	negligible	negligible	40	1. Human chromosome 14 clone RP11-463C8 containing POMT2 gene 2. Human chromosome 14 clone RP11-493G17 and CTD-2516D11 map 14q24.3	180,331 210,204	23/24 23/24
69	7-4	800	fwd7,4,2,a *	668	83.3	18	52	1. Helicobacter pylori 26695 section 116 of 134 of the complete genome 2. Drosophila virilis hook protein (hk) gene	9,956 4,054	40/40 40/40
70	7-4	800	fwd7,4,4,a *	562	negligible	negligible	9	1. Human BAC clone GS1-405L21 from 5p15.2 2. Human PAC clone RP4-539M6 from 22	235,141 159,593	20/20 20/20

Table 5.1g: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

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No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
71	7-4	800	fwd7,4,6,a *	531	negligible	negligible	25	1. Human DNA sequence from clone 30M3 on chromosome 6p22.1-22.3. 2. Botrytis cinerea strain T4 cDNA library under conditions of nitrogen deprivation	102,200 1,320	25/26 22/22
72	7-4	800	fwd7,4,7,a	380	negligible	negligible	21	1. Human DNA sequence from clone 37C10 on chromosome 1p35.2-35.21 2. Arabidopsis thaliana genomic DNA, chromosome 3, BAC clone: F3H11	216,497 33,442	20/20 20/20
73	7-4	800	fwd7,4,8,a *	770	55.2	105	40	1. Oryza sativa subsp. japonica BAC clone 34K24 1. Oryza sativa BAC clone 1.H19	142,852 77,605	44/44 44/44
74	7-4	800	rev7,4,1,a	880	negligible	negligible	51	1. Borrelia burgdorferi fla gene, isolate UK-Sco 2. Human DNA sequence from clone 305B16 on chromosome Xq25-26.2.	456 109,524	23/24 21/21
75	7-4	800	rev7,4,15,a *	524	negligible	negligible	36	1. Drosophila melanogaster genomic scaffold 142000013386050 section 44 of 54 2. Wolbachia endosymbiont of Trichogramma evanescens outer surface protein precursor gene	286,418 558	28/30 20/20
76	7-4	800	rev7,4,2,a	810	81.0	21	20	1. Human chromosome 17, clone hRPK.271_K_11 2. Human keratin (psi-K-alpha) pseudogene	199,182 9,697	21/21 21/21
77	7-4	800	rev7,4,4,a *	900	60.9	69	29	1. Human chromosome 14 clone RP11-463C8 containing POMT2 gene 2. Human chromosome 14 clone RP11-493G17 and CTD-2516D11 map 14q24.3	180,331 210,204	23/24 23/24
78	7-4	800	rev7,4,6,a	889	negligible	negligible	60	1. Homo sapiens chromosome 17, clone hCIT.22_K_21 2. Arabidopsis thaliana chromosome II section 193 of 255	135,259 88,411	44/44 48/50
79	7-4	800	rev7,4,7,a	643	61.7	60	21	1. Human DNA sequence from clone 37C10 on chromosome 1p35.2-35.21 2. Arabidopsis thaliana genomic DNA, chromosome 3, BAC clone: F3H11	216,497 33,442	20/20 20/20
80	7-5	800	fwd7,5,12,a *	653	negligible	negligible	28	1. Human chromosome 21 segment HS21C049 2. Bacteriophage A118 hol118 and ply118 genes	340,000 2,118	44/44 40/40

Table 5.1h: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
81	7-5	800	fwd7,5,14,a	816	negligible	negligible	88	1. Caenorhabditis elegans cosmid R11A5 2. Gallus gallus HMG2a mRNA	26,671 1,073	24/24 23/23
82	7-5	800	fwd7,5,15,a *	121	negligible	negligible	13	1. Mus musculus T cell receptor gamma locus, TCR gamma 1 and gamma 3 gene clusters 2. Drosophila melanogaster genomic scaffold 142000013386054 section 20 of 35	105,726 307,017	20/20 18/18
83	7-5	800	fwd7,5,17,a	848	negligible	negligible	37	1. Human chromosome 17, clone hRPC.159_F_22 2. Human chromosome 21 BAC B318B17 map 21q11.2	91,842 171,915	21/21 20/20
84	7-5	800	fwd7,5,2,a *	275	negligible	negligible	26	1. Mus musculus phosphoglycerate kinase (B24) pseudogene 2. Human DNA sequence from clone 798A17 on chromosome 1q24	2,138 130,467	20/20 19/19
85	7-5	800	fwd7,5,8,a	705	66.7	39	63	1. Human BAC clone GS1-589P19 from 7p13-p14 2. Melanoplus sanguinipes entomopoxvirus, complete genome	148,915 236,120	23/23 23/23
86	7-5	800	fwd7,5,9,a	315	62.5	80	13	1. Cinchona pubescens rps16 gene, chloroplast gene 2. Human BAC 161A6	763 100,364	20/20 20/20
87	7-5	800	rev7,5,10,a	601	68.8	32	31	1. Human clone GS1-278J22 2. Drosophila melanogaster genomic scaffold 142000013386055 section 24 of 63	152,517 261,329	21/21 20/20
88	7-5	800	rev7,5,12,a	497	negligible	negligible	28	1. Human chromosome 21 segment HS21C049 2. Bacteriophage A118 hol118 and ply118 genes	340,000 2,118	44/44 40/40
89	7-5	800	rev7,5,14,a *	531	negligible	negligible	11	1. Human Chromosome 16 BAC clone CIT987SK-A-363E6 2. Human Xp22 BAC GS-551O19	220,633 151,750	20/20 20/20
90	7-5	800	rev7,5,15,a *	657	negligible	negligible	32	1. Cynodon dactylon chitinase (Cht2) gene 2. Mus musculus Ena-vasodilator stimulated phosphoprotein (Evl), mRNA	1,788 1,791	42/42 40/40

Table 5.1i: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
91	7-5	800	rev7,5,17,a	653	negligible	negligible	37	1. Human chromosome 19 cosmid F15386, genomic sequence 2. Arabidopsis thaliana chromosome 1 BAC F5D14 sequence	38,000 127,462	21/21 20/20
92	8-2	300	fwd8,2,12,a	281	66.7	93	42	1. Arabidopsis thaliana chromosome II section 245 of 255 2. Drosophila melanogaster genomic scaffold 142000013386050 section 30 of 54	102,229 284,386	29/30 23/23
93	8-2	300	fwd8,2,14,a	520	negligible	negligible	47	1. Human pescadillo (zebrafish) homolog 1, containing BRCT domain (PES1), mRNA 1. Human PAC clone RP1-56J10 from 22	2,257 101,720	78/95 78/95
94	8-2	300	fwd8,2,18,a *	547	negligible	negligible	43	1. Drosophila melanogaster genomic scaffold 142000013386035 section 61 of 105 2. Danio rerio pes mRNA	260,278 2,214	21/21 33/37
95	8-2	300	fwd8,2,3,a	536	negligible	negligible	16	1. Human chr3 BAC RP11-64O13 2. Human PAC clone RP5-1008N9 from 7p21-p22	173,907 172,366	40/40 40/40
96	8-2	300	fwd8,2,4,a	490	negligible	negligible	8	1. Drosophila melanogaster genomic scaffold 142000013386053 section 9 of 30 2. Equus caballus DNA, microsatellite TKY276	298,939 563	23/23 22/22
97	8-2	300	fwd8,2,5,a *	260	negligible	negligible	23	1. Human DNA from chromosome 19, cosmid F21856 2. Human 3 BAC RP11-77I5	39,631 168,812	24/25 20/20
98	8-2	300	fwd8,2,5,b *	260	negligible	negligible	16	1. Human 3 BAC RP11-77I5 2. Caenorhabditis elegans cosmid C09F12	168,812 16,919	20/20 20/20
99	8-2	300	fwd8,2,6,a *	556	negligible	negligible	65	1. Human KIAA0575 gene product (KIAA0575), mRNA 2. Human DNA sequence from clone RP6-24A23 on chromosome Xq22.2-23.	5,285 137,100	19/19 19/19
100	8-2	300	rev8,2,12,a	194	negligible	negligible	8	1. Human clone RP11-9O7 2. Human clone RP11-78I14	157,067 179,357	21/21 18/18

Table 5.1j: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe Percentage	Similarity with probe Overlap	Hits	Sequence	Length	Extent
101	8-2	300	rev8,2,14,a	535	negligible	negligible	30	1. Human chromosome 16 clone RPCI-11_480G7	218,892	22/22
								2. Drosophila melanogaster genomic scaffold 142000013386050 section 6 of 54	276,243	20/20
102	8-2	300	rev8,2,18,a	565	negligible	negligible	40	1. Plasmodium falciparum MAL3P4	113,880	42/42
								2. Human Chromosome 16 BAC clone CIT987SK-A-954B10	118,521	40/40
103	8-2	300	rev8,2,3,a	160	negligible	negligible	40	1. Caenorhabditis elegans cosmid Y58A7A	30,781	42/42
								2. Human clone UWGC: y19c116 from 6p21	37,041	48/50
104	8-2	300	rev8,2,4,a	671	65.2	46	12	1. Drosophila melanogaster genomic scaffold 142000013386054 section 33 of 35	299,786	26/27
								2. Human mRNA for type 3 inositol 1,4,5-trisphosphate receptor	8,404	25/26
105	8-2	300	rev8,2,5,b *	441	49.3	217	21	1. Human 3 BAC RP11-771S	168,812	20/20
								2. Human chromosome 4 clone C0478G20 map 4p16	172,568	20/20
106	8-2	300	rev8,2,6,a *	315	negligible	negligible	28	1. Human DNA sequence from clone 790B6 on chromosome 20p11.22-12.2.	106,179	25/26
								2. Human chromosome 17, clone hRPK.293_K_20	185,254	21/21
107	8-3	300	fwd8,3,5,a	461	62.7	51	17	1. Neisseria meningitidis serogroup B strain MC58 section 71 of 206	10,982	22/22
								2. Human replication protein A2 (32kD) (RPA2) mRNA	1,512	22/22
108	8-3	300	fwd8,3,6a *	320	negligible	negligible	30	1. Leishmania sp. isolate PP19 kinetoplast minicircle-k sequence	722	19/19
								2. Waddlia chondrophila 16S ribosomal RNA and 23S ribosomal RNA	4,785˙	19/19
109	8-3	300	rev8,3,5,a	488	negligible	negligible	17	1. Synthetic gene containing TMV Omega-prime leader and NPTII mRNA	121	23/24
								2. Arabidopsis thaliana chromosome II section 148 of 255	115,175	22/23
110	8-3	300	rev8,3,6,a	371	negligible	negligible	38	1. Canis familiaris TCTA gene, AMT gene, DAG1 gene and BSN gene	162,073	22/22
								2. Arabidopsis thaliana DNA chromosome 4, contig fragment No. 33	190,026	23/24

Table 5.1k: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
---	---	---	---	---	Percentage	Overlap		Sequence	Length	Extent
111	10	600	10_13	376	negligible	negligible	35	1. Mus musculus hepatocyte growth factor activator inhibitor type 1 2. Bacterial sp. gene for 16S ribosomal RNA	2,228 202	20/20 20/20
112	10	600	10_53f	407	60.4	48	36	1. Arabidopsis thaliana chromosome II section 243 of 255 2. Human chromosome 17, clone hRPC.1171_1_10	77,999 175,120	21/21 23/24
113	10	600	10_63r	385	91.7	24	71	1. Mus musculus leptin (Lep), mRNA 2. Meleagris gallopavo leptin precursor mRNA	504 438	20/20 20/20
114	10	600	10_10R_rtt *	808	negligible	negligible	21	1. Human BAC clone RP11-304C24 from Y 2. Caenorhabditis elegans cosmid Y73B6BR	202,354 11,959	21/21 23/24
115	10	600	10_5R_rtt *	565	negligible	negligible	48	1. Campylobacter jejuni NCTC11168 complete genome; segment 4/6 2. Human BAC clone RP11-304C24 from Y	282,183 202,354	43/43 42/42
116	10	600	10_15R_rtt	943	61.4	57	6	1. Drosophila melanogaster genomic scaffold 142000013385248 2. Human clone RP5-1070G24	24,208 157,075	22/23 22/23
117	11	1500	11_20F_rtt *	828	81.3	32	62	1. Gallus gallus GART-B gene, exons 11-22 2. Chicken neuropeptide Y gene, exons 1-3	18,348 6,423	33/35 31/33
118	11	1500	11_20R_rtt *	677	69.0	42	8	1. Human Chromosome 16 BAC clone CIT987SK-A-923A4 2. Genomic sequence from Mouse 4 (Accession: AC002108)	82,883 41,125	20/20 20/20
119	11	1500	11_23R_rtt *	651	81.3	32	65	1. Gallus gallus GART-B gene, exons 11-22 1. Chicken neuropeptide Y gene, exons 1-3	18,348 6,423	33/35 31/33
120	11	1500	11_24R_rtt *	846	69.0	42	33	1. Chicken glutamine synthetase gene sequence 2. Gallus gallus class II cytokine receptor gene cluster	2,667 46,304	28/28 25/25

Table 5.1l: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

| No. | Clone | Band size (bp) | File | Length (bp) | Similarity with probe | | Hits | Homology with known sequences | | |
					Percentage	Overlap		Sequence	Length	Extent
121 1	11	1500	11_25F_rtt *	741	69.0	42	9	1. Mus musculus Cast (Cast) and peroxisomal membrane anchor protein (Pex14) genes 2. Human Chromosome 16 BAC clone CIT987SK-A-923A4	53,094 82,883	26/27 20/20
122	11	1500	11_25R_rtt *	651	81.3	32	29	1. Gallus gallus GART-B gene, exons 11-22 2. Chicken neuropeptide Y gene, exons 1-3	18,348 6,423	33/35 31/33
123	11	1500	11_26R_rtt *	814	negligible	negligible	11	1. Human 3p21.1-9 PAC RPCI5-966M1 2. Human inter-alpha-trypsin inhibitor family heavy chain-related protein (ITIHL1) gene	133,475 1,173	22/22 22/22
124	11	1500	11_27R_rtt *	826	87.5	16	31	1. Drosophila melanogaster genomic scaffold 142000013386035 section 44 of 105 2. Human BAC clone GS1-587J1 from 7p12-p14	218,434 98,433	20/20 20/20
125	12	2500	12_26F_rtt *	779	87.5	16	34	1. Drosophila melanogaster genomic scaffold 142000013386035 section 44 of 105 2. Human BAC clone GS1-587J1 from 7p12-p14	218,434 9,8433	20/20 20/20
126	12	2500	12_28R_rtt *	894	57.1	63	34	1. Drosophila melanogaster genomic scaffold 142000013386035 section 44 of 105 2. Human BAC clone GS1-587J1 from 7p12-p14	218,434 98,433	20/20 20/20
127	12	2500	12_29F_rtt *	850	negligible	negligible	11	1. Human 3p21.1-9 PAC RPCI5-966M1 2. Human inter-alpha-trypsin inhibitor family heavy chain-related protein (ITIHL1) gene	133,475 1,173	22/22 22/22
128	12	2500	12_29R_rtt *	838	87.5	16	33	1. Human BAC clone GS1-587J1 from 7p12-p14 2. Human DNA sequence from clone 403L10 on chromosome 6q25.1-26	98,433 60,215	20/20 23/24
129	12	2500	12_30_16F_rtt *	651	72.0	25	10	1. Human 3p21.1-9 PAC RPCI5-966M1 2. Human inter-alpha-trypsin inhibitor family heavy chain-related protein (ITIHL1) gene	133,475 1,173	22/22 22/22
130	12	2500	12_30_16R_rtt *	707	negligible	negligible	10	1. Human 3p21.1-9 PAC RPCI5-966M1 2. Human inter-alpha-trypsin inhibitor family heavy chain-related protein (ITIHL1) gene	133,475 1,173	22/22 22/22

Table 5.1m: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
131	12	2500	12_30_18F_rtt	735	negligible	negligible	12	1. Human 3p21.1-9 PAC RPCI5-966M1 2. Human inter-alpha-trypsin inhibitor family heavy chain-related protein (ITIHL1) gene	133,475 1,173	22/22 22/22
132	12	2500	12_30_18R_rtt *	637	57.1	63	31	1. Human PAC clone RP5-819O4 from 7q33-q35 2. Drosophila melanogaster genomic scaffold 142000013386035 section 62 of 105	124,293 238,433	21/21 20/20
133	12	2500	12_30R_rtt *	636	negligible	negligible	15	1. Human 3p21.1-9 PAC RPCI5-966M1 2. Human inter-alpha-trypsin inhibitor family heavy chain-related protein (ITIHL1) gene	133,475 1,173	22/22 22/22
134	14	2500	14_31F_rtt *	320	negligible	negligible	54	1. Chicken mRNA for nuclear b-Zip protein MafF 2. Mus musculus gene for mafF	1,800 2,699	193/197 162/197
135	14	2500	14_31R_rtt *	608	negligible	negligible	14	1. Chicken mafF gene, 5'flanking region 2. Drosophila melanogaster genomic scaffold 142000013386053 section 6 of 30	706 302,915	443/455 22/22
136	14	2500	14_32F_rtt *	618	negligible	negligible	109	1. Chicken mafF gene, 5'flanking region 2. Leishmania chagasi (clone C1) ribosomal protein P0 gene	706 1,373	268/314 27/31
137	14	2500	14_32R_rtt *	311	67.6	37	53	1. Chicken mRNA for nuclear b-Zip protein MafF 2. Homo sapiens v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G (MAFG) mRNA	1,800 1,677	184/189 118/142
138	14	2500	14_33DR_rtt *	682	negligible	negligible	37	1. Chicken mRNA for nuclear b-Zip protein MafF 2. Mus musculus gene for mafF	1,800 2,699	202/206 171/207
139	14	2500	14_33F_rtt *	721	negligible	negligible	24	1. Chicken mafF gene, 5'flanking region 2. Drosophila melanogaster genomic scaffold 142000013386047 section 47 of 52	706 234,905	419/448 20/20
140	14	2500	14_33NR_rtt *	336	78.3	23	64	1. Chicken mRNA for nuclear b-Zip protein MafF 2. Human v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G (MAFG) mRNA	1,800 1,677	186/189 119/142

Table 5.1n: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

No.	Clone	Band size (bp)	File	Length (bp)	Similarity with probe		Hits	Homology with known sequences		
					Percentage	Overlap		Sequence	Length	Extent
141	14	2500	14_33R_rtt *	340	negligible	negligible	61	1. Chicken mRNA for nuclear b-Zip protein MafF	1,800	185/189
								2. Homo sapiens v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G (MAFG) mRNA	1,677	119/142
142	14	2500	14_34F_rtt *	740	negligible	negligible	11	1. Chicken mafF gene, 5'flanking region	706	424/446
								2. Drosophila melanogaster genomic scaffold 142000013386047 section 47 of 52	234,905	20/20
143	14	2500	14_34R_rtt *	340	52.7	74	64	1. Chicken mRNA for nuclear b-Zip protein MafF	1,800	185/189
								2. Human v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G (MAFG) mRNA	1,677	119/142
144	14	2500	14_35R_rtt *	341	negligible	negligible	62	1. Chicken mRNA for nuclear b-Zip protein MafF	1,800	185/189
								2. Human v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein G (MAFG) mRNA	1,677	119/142
145	14	2500	7285 *	370	negligible	negligible	20	1. Human Bassoon (BSN) gene, exons 6 to 11	6,560	21/21
								2. Human gene for 24-kDa subunit of complex I, exon 7	2,362	20/20

Table 5.10: Sequences from the tertiary screening of the chicken genomic library in the cosmid vector selected for making consensus. These sequence files were entered in the fragment assembly system of the GCG programme after removing the vector sequences to make consensus sequences. Asterisk mark denotes inclusion in a multi-fragment consensus as shown in table 5.3 a-g. Sequences related to leptin are printed in red and sequences from the chicken are printed in green.

Serial No: 1	Cosmid clone(s): 10	Consensus length: 560

contig fragment assembly

```
      3   10_5r_rtt    <-------------------------------+
      2   10_10r_rtt +-------------------------------------->
      C   CONSENSUS  +---|---|---|---|---|---|---|---|---
                     0   200   400   600   800
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

```
tmpseq_1  +---|---|---|---|---|---|
          0   100   200   300   400   500
```

Consensus sequence

```
  1   AAATGAATGC AGCAGTCTGC TCTGTGGCCG AGCAGCATCA CGTTCTTTGT GGTyTCATGT AGTAGTAACA ATCCTACCTG TATTACATGT GGATTGGATA TCCAGTTGCT aAAACAGTTA
121   GAGACTGCTA CTTCAGAAAA ATGCTTTCAG GCGCCAGAaA GTCAGATTAC AGTAATTTgT TACACAAGAA TGCAACTGAA AATCAGAGTT CTGTTAATTT ACTTGTCCCT CCTCAAGCCC
241   TCTCCCCATT TTGCTGGCCC AAGTATCTAT GTTGTTATTT TTTTTTCTCC CCAGGAAAAA AGTGTTAACA AAAGACACAA CAAGAGaTCA TTAATAACTA TCATCCATAG
361   AGAATACGAG CAAGCAGCTG TCAAACCATG CCAGACAAGAC TGAAGTCTCA CCAGGACTGA GTTCTGCATC CCCAGTGTTA CAAACCCTCG TTTCAAGTGT TTGGgTTTTT
481   TAAAGTAACT TACAAAAACT AATTTCAAAC ATCACTCAAT ACTAGAAGTA AGAGCACCAT TTCTACTAGT TCAATCACCw
```

Hits	Sequence	Length (bp)	Extent
27	1. Homo sapiens BAC clone RP11-304C24 from Y	202,354	21/21
	2. Caenorhabditis elegans cosmid Y73B6BR	11,959	23/24
	3. Drosophila melanogaster genomic scaffold 1420000133386050 section 3 of 54	287,649	23/24
	4. Human Chromosome 15q26.1 PAC clone pDJ417d7	115,888	20/20

Table 5.2a: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

| Serial No: 2 | Cosmid clone(s): 11 | Consensus length: 1585 |

contig fragment assembly

```
7   11_20r_rtt    <------------+
6   11_25f_rtt    <------------+
5   11_24r_rtt    <------------+
4      11_25r_rtt +------------->
3      11_23r_rtt +------------->
2      11_20f_rtt +------------->
C   CONSENSUS  +------|------|------|------|------>
              0    400   800  1200  1600
```

Homology

Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

tnpseq_1

(positions) 0   250   500   750   1000 1250 1500

Consensus sequence

```
1     CAGCTTCTTT GCATTATTGA AAAATGTTA ATAGTCATGT GTGTATATAT GtATTtatAG GTAGAGATG CTGCCGTGAG AtAAGCCACA AAATGGTTCA AAGCAGAATC ATAGAATCAC
121   TGAGGTTGGG AAAGACCTTC AAGATCCCCT AGTCCAGCTG TTCACCTGCC ACCAATACTT CCCACTAAA CTATGTCCCT CAGTGTATGA GTTCTGGGTG GGATAACCTT GCTTTTGCTC
241   AGGGAGATAT GTTATTAGAT GACTAATTCT TCTGGTGGAT GATAACACGG TATTTGCtGT CTATGTATGA AGCAGTAGAG CGGaCTGAGG TGTCAAGCCA
361   GTTTTGGGGG AGAATGTGCC AACTTCCTTC TTGGGCTGTC GCCTGCATCT GTGATGCTC CTCCTCTTGG AGGGTGCG TGTTTCCGCG TtGCCATCAG
481   GCGCTGCCTT CGGGGGACGG TGGAGGCAgA TTCAGTGTCA CAGGCAGCCT TCCAGCCAGG CAGTAGTGGA AACCTCCTCG AGGATCATCT GCTCCGCCTC TCCCTTGCCT
601   CTGAGCTATT TGTGGCtTcc ACAGATATGA AAATAACCTA TTkGgtGGG GGTGTTTTG CTGCTATCAA GAAGAATGGG GATTTGTTTG TCTTTTGATA GATGATCTTT GGTTGGTTGG
721   TTGATGGTTT CTTTTTGAAT AGaAagCTGg TgGGCTTTGA CCCTgGAAGC aATCAArGCT GGsCCTGGGC AgCCTgGAtGc gGTGAgtArC AGCTCAGCCT
841   aTGGCAGGGC TGrAACCTGg TgGGCTTTGA GTCCCTTGCA ACCTATGATT CTATTATTGT AGTCATCAT TTcCCCCcA TTACTTTAAA TGTCGGCTTT GCAGGTAGTC
961   TTcTGGCAGT AAAGCTCTTT CCCCTGCCAC TGGGACTGGC TGGCTGTTCC TAAACAAACA GCCAGCTGCA TAGTGACAGG ATGGGCCAGT ACTTAGTCCA CACCGGTGCC
1051  TTcTGGCAGT AGGAAGTTCA CAAACCAGCA TTGCTTTCCT TAGGCTCTAT CCATGAGAAG AGACAATAAG AGCTATTCGG AGTGAATGAG GAGGCACTCA
1201  GTGGGTGATG CAAACCAGCA TTGCTTTCCT TAGGCTCTAT CATGCATGCT GTTACTTCG GGGGTTGAT GCAGCATGCA TTCCACCTGG TAGAAGCTAG CGTACCTTG
1321  GTCTGGTGTT CCAAGCAGTG CCATGAGAAG GCAAATGCTT ATTTATTTGG AGGAGCGAGC TGATCTCGTG AGCTATTCGG AGTGAATGAG GAGGCACTCA
1441  CCTTGCAGCA GGGGGATGTG GCAAATGCTT CCCATAACCG TTTCCGTCAG AACAGAGTCC TGTTACAGAA CCACTCCTGC TCCCTGCCAA CCTGCCTTTT TTTTTCTGCT TGCTCCTCTC
1561  CTTCCTACTC ACTgCCATTG TCTCT
```

Hits	Sequence	Length (bp)	Extent
75	1. Gallus gallus GART-B gene, exons 11-22	18,348	33/35
	2. Chicken glutamine synthetase gene	2,667	27/28
	3. Chicken neuropeptide Y gene, exons 1-3	6,423	31/33
	4. Meleagris gallopavo clone TUCA883 microsatellite sequence	310	30/32

Table 5.2b: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for Cor G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 3 | **Cosmid clone(s): 3, 5, 7, 11 and 12** | **Consensus length: 2,277**

Homology

Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | X>=200 |

contig fragment assembly

```
      12   fwd7,5,2,a            +----->
      11   rev5,1,5,b          +------->
      10   3_2_7                 +----->
       9   rev3.2,14,d           +----->
       8   rev3.2,14,a           +----->
       7   12_30_16f_rt          +------->
       6   12_30_16r_rt          <-------+
       5   12_30r_rtt            <------+
       4   12_30_18f_rt          <------+
       3   11_26r_rtt            <------+
       2   12_29f_rtt            <-----+
       C   CONSENSUS          <----|
           |------|------|------|------|------|
           0     600   1200   1800   2400
```

Consensus sequence

```
1    TmmTCCACAC GGGAGATCTC CAACCGYTAC TCCGGsGAGd ArAGAcKTAC AAGTCGgrrA rGTCTCCAAT tCC.TCTACA TCAATGGCGA CytGCGTAAG AGTGAGAAGG TGAAGATGGA
121  CATCTGTGGg AatgTGACCA CCAACAATGA GAAGAACATG CCCAGTACCG AGagCCCAGT AaCCC.ACCA AAGATATTgC cAATcTCCGG TTACTaTGTA GaTGGtGAGT TTTATTTaC
241  CTCCTGGCTC GTGATGGTTG TGGCTTGGCT CTAtaAGCAC AAGCCGGGCA ATGGTGTAAT CCTcACAaaT AGtCTGACTC AGtTCTTCA ATCATTTAAC
361  AGTTCATATA ATATTT.aCT ACTTTCATGG GGCAAA.GGA GAATGCTAGT TCTGAGAGCA TAATAATAAG TCTTTCAGAG CTAAGCTTAC AGTTCTTCA ATCATTTAAC
481  CCACCTGAAT TGCTGTGCCT ATCTCTCCTA TTGACAGAGG GGAAAGTGG AGCTGGCTCT CTTTTGGTC AGCCAGAGCC GATGAGGCTT GGGGCGTTT ACAGACCATC
601  TCCTACAGCC CACTGCCACA AAAAGGTCTT ACTTGAATGA AGTTGTGTGT ATCTGTTCAA AAGCCTAGTG CTCCTGGTGA GGGGCAATCC GTAAACTACC CCTGCAACA
721  TTATTCCCTG TCCAAGCACA GTCCTCATGG CTCATATCGG CTTAGAGGAT GCCCAGAGCC CAGATGTTTT ATGCAGCCTG CTGCTCTGCT CTGTAATTTG TGTCCTG...
841  GgGAcgCTGC CAGAAGCAGc gtcGTTwmTG CAGCTTTGCw GCAgsGCTGC ArtCCATTAC AGTAGCTTTG vtGATGGAAT CCAGCAGATC TAATAGTTGT TGTTTTGACT
961  GAgGTGTTGG CAACTTGAGG CTTTTATAAA GACTACTGCA AGACGGGAAC TTGTTCAgTT TTCTTCTTT GCTTTACCGA ACATATTCCT GTCTCCACAT GCTCTAACCG
1081 TTGATTTTTT TCATGCCTGT CTGTATGAAA gCATCCTGTC CGTCAagTGT CAAAGACTCTG TCTTGCTCTg GGTGTTGgAT ACAGAAGTat CCTGGTGACT GACTGCGTTT
1201 CAATATTTAt TtCTTCTTCC tctGAAAGCA GCATAATAAA GTCAAgTGCT AACGCACCCC TCTTGCTCTg GGTGTTGgAT yTgtGtgACT TC.tCTGcCt CAGCAgCCTg
1321 AGCCGTCCTC GCTCCCACCT CCTCCTCT.G ctgGCCT.CC ccatGGTGGC GCTGCGCcat atgtCCGCat a.GaaTgTca gttgaacd tttTcccCCa
1441 CggtaCAAT tcAtcttcAg TttAAtgCct tCgtaGagaa aTagctAcAG a.GaaTgTca gctGCgCcat atgtCCCttc catataaca gttgaacd tttTcccCCa
1561 AGCCTkGCCG CGTAGGGCAC kCAAGAkAGG AGsAGAkGCA TATGAAAGCA GAdCCAATGA AAACsTbCAT TGTWAGyAAw ksCCGyAGAG rCrCTTGATG GTACAGCCCb sGGGGGGGCk CAGGCCGCCC
1681 AGCCTkGCCG CGTAGGGCAC kCAAGAkAGG AGsAGAkGCA TATGAAAGCA GAdCCAATGA AAACsTbCAT GCCTCGGGGC AAGGCTCGGG GGrAAGCACC ACGGCGdGGC yAATACCsCC
1801 ATyskkGAG GswGGkAGCA TCGTCGGKGG ACrCAGCACr TCsCAGCCTT TTrGCTGGrs GkrGTGAGAG sACAGTCCCT ACGGCGdGGC GGArACdCAC GTTkCCGCTG
1921 CwGCCrATCy GsCTCGwrAA rGGGsCkCGG GACGAAACrA rGCdkGAGCd GGsrTGGCwC WGCACyTTsA GGaCaACACA ATCTGTGCCT GACTACACAG ACACTGCTCC
2041 ATGATGATGC ACGCTGGTGT ATTACTGCTG CACGTCTGGT GTGCTCTGTC TTGGGAATCG TTGCTTGAGC CCTTTCCTAC GTGGTTTTGT GTTGTAAGGA CTCGCAGAGA
2161 CATTAACAGA CGTCGGATAG TTGATGGTAG TTGATGTGTAG GAAAAGGGCG ACAAACCACC GTGGACGGCA GAAACGACGC AACAGAAGAA AGGATAATGA AAAAGCTGTA AGGGGT
```

Hits	Sequence	Length (bp)	Extent
45	1. Human 3p21.1-9 PAC RPCI5-966M1	133,475	22/22
	2. Human inter-alpha-trypsin inhibitor family heavy chain-related protein (ITIHL1) gene	1,173	22/22
	3. Human clone RP5-1193P9	133,991	21/21
	4. Human DNA sequence from clone RP4-742C19 on chromosome 22	122,748	21/21

Table 5.2c: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambiguous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

143

Serial No: 4	Cosmid clone(s): 14	Consensus length: 822

contig fragment assembly

```
        5    14_31r_rtt   +----------------->
        4    14_34f_rtt   +----------------->
        3    14_33f_rtt   +----------------->
        2    14_32f_rtt   +----------------->
        C    CONSENSUS_   +----------------->
              |----|----|----|----|----|----|
              0   200  400  600  800
```

Homology

Color Key for Alignment Scores

tmpseq_1

Consensus sequence

```
  1  CGsdvGkGGG GvGnrkGGGA AArAAACCCC CnCGnGGTCG mGCGGCkmTC GATnAGCTTG nTnTCGnATT CCTGCAGCAC CGttCTATCA GGCCtCGC.t GCg.g.GCCC CG.aGGGGaC
121  aaCTGagGCC GCAGCACCTA CCGGAAggcc ACCCCCGGGC TCTGGGGGtT GACCAGCaCG CCGTCCCaGC aGCGGCCGTT CCCGTaGAGC CGCaCCCGGC CGTCCTCCGT CAGGCGGCTC
241  TGCCCCGaGT ACTCGGCGGC GGaCaCCTCC TTCaCCCGGT aCGT.TG.CT GaACGGCTGG CTCaAaAGCC cCacaGCTCC CTGcaTGGCa CGCG.GCTCC CCGGCTCGaC CTTGaGaGaG
361  aCaaCCGaCG aCTGaCGGGC CGGaGCCGGC aCTTTTGGTT TCTGTACCGC CTTCCCCTTA CGaGaaGGGC aGCCGCTTCT CCacaGCTCC CGc..GGGcC TCaGGGaAAC CGaGGCCCGc
481  aGaTCCCaCC GCCCTCGaTG CCCCAGAGCG AACCAATCAG CTGCGctGcG GGAGTGGCCa ATGGGGAAGG GCCAgTGGGG AgGGGCCAAC GGGGAGGGGC CAATGGGAG
601  GGGCCAgTGG ACTCCATTC CAGAGAAGGG TTGAAGGAG GAgCCGGCCG CCGCCATCTT TGAAAAgggc GGagGGgCGg GcGggGcGGg gCGGGGCGGG GCGGGGGCGG
721  GCGGGGCCCC CCGGGGCGGG GCGAgAGGaA gGTGA.GTCa CGCmTGcmTG TgTawwwAwa AwrAAAgAAw csCsGGtrAg tATCGCGCGA GATCGCTCGm rC
```

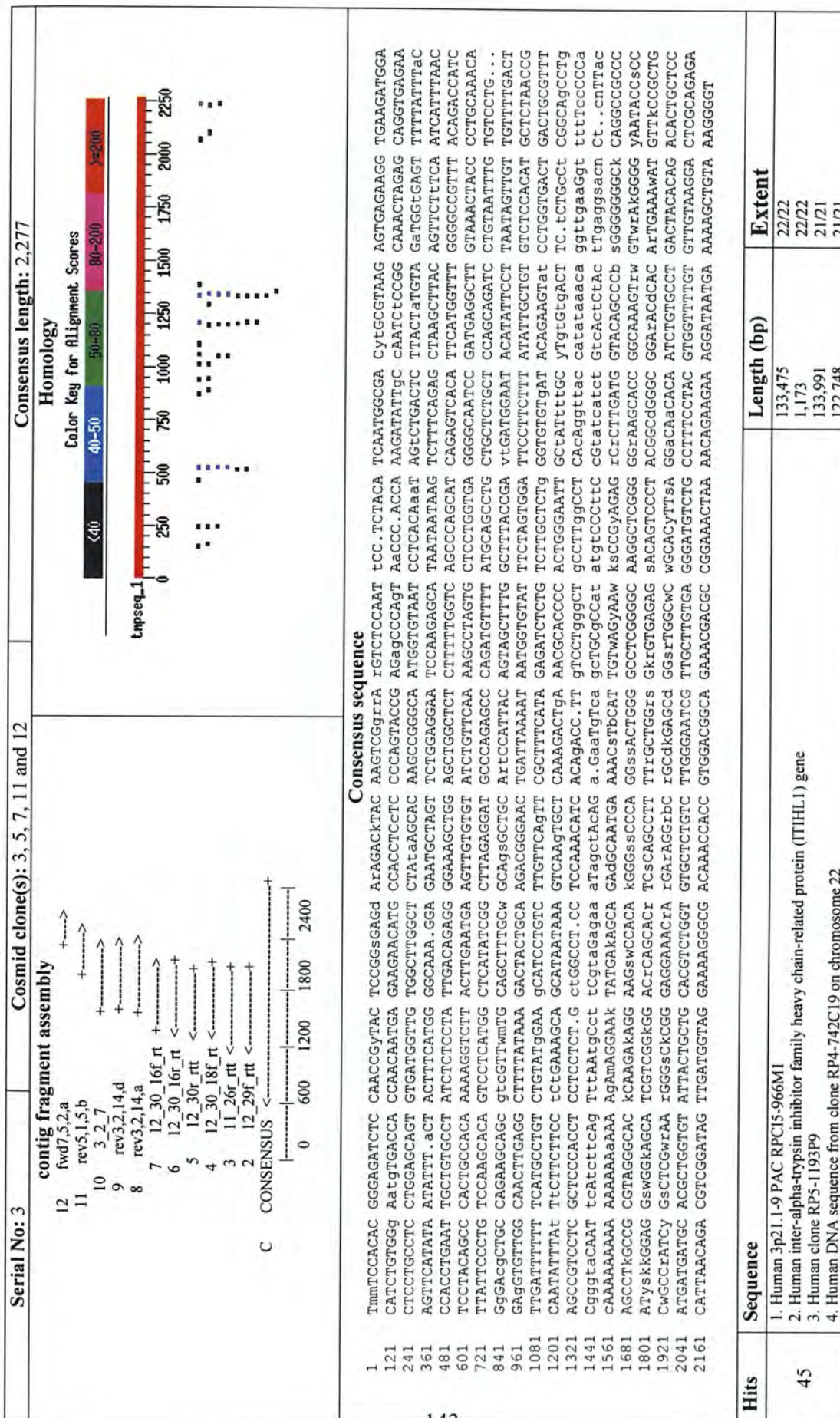| Hits	Sequence	Length (bp)	Extent
15	1. Chicken malF gene, 5'flanking region	706	442/454
	2. Drosophila melanogaster genomic scaffold 142000013386053 section 6 of 30	302,915	22/22
	3. Drosophila melanogaster genomic scaffold 142000013386047 section 47 of 52	234,905	20/20
	4. Human BAC clone RP11-4N7 from Y	135,049	20/20

Table 5.2d: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 5	Cosmid clone(s): 2	Consensus length: 909

contig fragment assembly

```
7  2_11            +------------------>
   6   2_1_10      +------->
   5    fwd2,1,10,c +------>
4   rev2,2,15,a    +------>
    3    7285.      +------->
2   2_2_5    +------->
C  CONSENSUS +------------------------->
         |------|------|------|------|
         0     200    400    600    800
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

tnpseq_1

Consensus sequence

```
  1  GCCCACTGGG AAGAGCATTT TGGAAAGAAC GGCTCAGTyT CACGCACCAA CGAGArATTC GGCrCACCCC CCAGGTyCCC CCArAACArC AGACTGAGCC GyCTGCATGG GTCyCTTTAA
121  AGAGCTTTyT yTTtggA.Th bcbaa.ktvc TgCAgcaGgG cccacCAGAG CAGCCAAGC. T.GcgA.gcA AAGCGC.A.A CTTGGGGTCC CACGTAGGAT GGGCGCTGCT GGGATcAGGC
241  CTCAGGTGCC CGCTGCGCGC AGGCTGTGGC TTGCCAGCAC TGCTGCACCC CCTGTTGTGC ACCTGTCTGG CCATGCACGC CTGTGTTCCT GGAGCAGCTG
361  cTGCCACTTC CCAGCACAG AGCTTTTGTg CCACCGGCGT CTGCTTTGCT GCCATGGGCA ATggaGACGG GAACTcAGCT CCAGGGATCT TgGGGGTTGG
451  sAGCaggagC CTkggagcTg ggggGCaATg AGGGACACGT CAgGGCTGGA TATTAGCAGC TGGGTGCTGG kGgaGCAACG AGGGATGGGT TGGGTTGGAT GTTCGGGTTG
601  GATATTGGGA AAGATTTCTT CTCCAAATAG CGGTTGGATG CTGGCACACT G.CCCAGGGG GTGGGGAGGT CACTGTCCCT GGGGATGTTC AAGGACTGTG GGGATGTAGA ACT.GGGGGA
721  TGTGGTCGGT GGGCAGCGGG GGGGTGAGGG GGATCTTTTC CAACCCCTTC TTTTTCTACG ACTCCCCTTC CAAATGGGAT TATGACAACA GCCAgAcAGG GGCgGCATCT Cagggawkkk
841  TkwkkwTwkT kGGGaCACGG AgcAmAsCCm AgrGccctGy CTTyTGsyGG GAGGGGAGCA CAGGATGGG
```

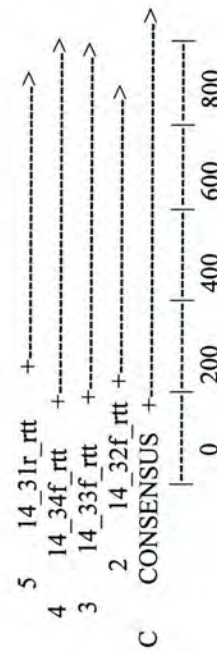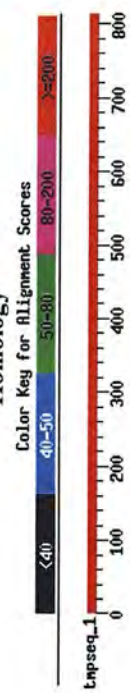| Hits	Sequence	Length (bp)	Extent
32	1. Pheasant ovotransferrin(conalbumin) gene frag. repetitive seq	739	40/44
	2. Chicken alpha-A-crystallin gene	6,311	33/35
	3. Meleagris gallopavo clone TUCA930 microsatellite sequence	314	32/35
	4. Gallus gallus rho-globin, beta-H globin, beta-A globin, epsilon-globin, and olfactory receptor-like protein COR3'beta	30,539	38/43

Table 5.2e: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No:6	Cosmid clone(s): 2	Consensus length: 1,027

contig fragment assembly

```
        rev7,1,10,a        <----------+
     3
        2   2_8     +----------------+
     C   CONSENSUS  +-------------------------->
         |------|------|------|------|------|
         0     400    800   1200   1600
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

tmpseq_1

```
|------|------|------|------|------|
0     250    500    750   1000
```

Consensus sequence

```
1    ATGGACAAGT GGCTCCCACC CCGAGGGCTG GGGAAGAGTG CTGTCCCATG AAGGAGGAGG CAGGAACCTG CTGTGAGAAA GCTTTTATTT TAAATAACCT CAAAAAAATA GAGTTGCAAG
121  GACCTGAAAT GTCGCTGGAG CATGATGCTG GGTGGAAGGA AGGGAGCGGG GCTGAGTGCT GGAGGGGGCA ATGCCACTCC CTGCCTGGGG ATGGAGCGGG GGCACCCATG CCAGGACGCC
241  ACCGCCCCTC GCGTGGTCGT GCCACCAGCT GGGCACTTTG CTAATGAAA ACATCCTTAC CCAGCGTGCA GGTCCAGCGG GACCATCCCG GTGTGCCTCG TGGCTGCTCC AAGCCCTGGA
361  GCTGAGCAGC TCTGGTTATT GTCTGCCCTA TCCCTGTCCA CACGCCCACT GCCCCAGCC GTGTGCCTTT GGTGTTGGA CATCTCCAGG AATGGAGACC CCACCAGTGC
481  TGGGCTGCTG GTGCAGTACT TCTCACTGCA CAACAGAAG TGTTCCCTTA TGCTACGCTC CGAGATATGC GCCAAAACAC AGGGGAGGAG GTGGTGGCAG GCAGAGCACA CCCAGGACTG
601  TGCCGCACCG TGCTAACAGT GCTAACAGT ATCCCACGCG AGGTCTTTGC CAAACTATTG GAAGCAAGGA GGCTCTkCCC CAAGGCTTTG TTTTTGATGT TTTTGCTGCT
721  CCAGCTTTCC CTGAATGCCC TAACAAATGA ATTTGCCGTC AGAGTGTTTA AAGGGAAAG ACAAATTTTA TAAAAATTGT GTCTGCATGA AAATAAATGC TTGATTTTTA TTTTTTTTTA
841  ATTGGAAATC TGATTTTCTG GATTAGTGCC CACCCAACCA GAGAGGAGAA GCAGATGCTT GGTGTATAAA CAAGCAATC AGGGCACTGA TTATTCAAAG GCAAAACCTC ATCACACTGC
961  AAATGGTTCA TAGAAATCAT CCAACAAATT CCTTCTGAAC ATCCAGCCTG TATAAACGCA CTGCCCT
```

Hits	Sequence	Length (bp)	Extent
47	1. Caenorhabditis elegans cosmid R10E4	36,170	22/22
	2. Human wingless-type MMTV integration site family, member 5A (WNT5A) mRNA	4,114	21/21
	3. Human DNA sequence from clone RP1-32I10 on chromosome 22	93,312	24/25
	4. Human proto-oncogene (Wnt-5a) mRNA	4,114	21/21

Table 5.2f: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 7	Cosmid clone(s): 3 & 5	Consensus length: 531

contig fragment assembly

```
      3   fwd5,1,5,b  <-----------------+
      2   3_1_6       +-------------------->
      C   CONSENSUS   +---|---|---|---|---|
                      0   200   400   600   800
```

Homology

Color Key for Alignment Scores

| <40 | 40~50 | 50~80 | 80~200 | >=200 |

```
t.npseq_1
          |-----|-----|-----|-----|-----|
          0    100   200   300   400   500
```

Consensus sequence

```
  1  TAGAAAAGA CAGGAATTAt TGATAGCAGa wGCAATGaAA AAygTGCaAT ttAAATcAAA GgCCTyAGAT ACACCATAGc GGAACCAGCC ctTTGGGTGG TTCTCATGCC TCCCATCCGT
121  TCCTCTCATG TCACTCAAGA TATGAAGCAc CCACAGTTTc CccCAGGcc ACGTTTcCCT CTTTCAAGGC TCTGGGAAAT CACCTCAAAG GAATTAAAgG GTGCAAACCC CCACTGTTA
241  GGCAGGGAGC AGCTCTTGGT ACTCAGCACT CCCAGCCTTA GAGCTTTAGG TAGTTAGATC ACATGCCCTA CGTCTGTTCG GAAACTCACA AGAAAAAGTC TTCCTCTTCA TCCGAATCCT
361  CCTCTAAAAG GTTCCTCTGT GAGCAAACAA AGAAAAGCAC CATCAGTATC AGCCATTTCA CATCACCTTC AGGACAGCAC CGGCACCTTC TGAGCACAGC GTACTGTGAG CCCTCCTGGG
481  aTaagCAGCA GTGCTgccTT TCAGTACCG GAGCTkTGCT kTGTTTCTCC C
```

Hits	Sequence	Length (bp)	Extent
14	1. Mus musculus leptin (Lep). mRNA	504	20/20
	2. Meleagris gallopavo leptin precursor (ob) mRNA	438	20/20
	3. Gallus gallus leptin precursor (ob) mRNA	492	20/20
	4. Mus musculus obese precursor (ob) mRNA	2,793	20/20

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Table 5.2g: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 8 | **Cosmid clone(s): 7** | **Consensus length: 888**

contig fragment assembly

```
3   fwd7,4,2,a              <------------------+
    2   7_30           +-------------------->
C   CONSENSUS  +----|----|----|----|----|----|
               0   200   400   600   800
```

Homology

Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

Consensus sequence

```
1    CTGCAGTCAG GATATCTCCC ATTTGTCTTC CTGCAAGGGG TAAGCTAAGT GCTCAGTGCT GCAAAAGGCA GCATGTATGG AGTAAGATGG ATTGTGTGAGC GGCAGGATGC TGGGCAGGAG
121  CCGCACTCGG TGCTTGCAGG GACGGGGGAG CAGGTGGGAC CCCCCAGTGG GCTGCAAGCA GGGTGyATT  cATcrTTcaT CAGGAtGrGy AGtwGkGkkG
241  GwACCACAGC ggTGGtTTGG rCAGcTwGwG CyAGcmwTky TGCTkTGywG TGGGGCTGTG kTkGtGGkGC rGmwCCkmGG tCTGcTGrrG GyAGCmGAAA
361  AAAatGGAGG tTGAGAGTGG cGGAGAAATG ACACACCTGT CCCATCAGAC CAGCAACTGA GAAGAGCCAA TTAGCATATT TTAATGTAAC TGATwArTGC
481  CTGGTCACAC AGCCCGCTTA gTgtGkTGAT GTTGGAAGCA TACAAGCATG CCCATAGCAA CCCACGAGGG AGCTGGTGGT AGGAGAGATG ATTTCCCATC TTAGCAGCTC
601  TGTCCCCATT TCTCTGTTAT TACCCGTGGA AAGCTGCCTT CAGAGAGCCC TGCCGTGGTC CGGGGTGAGG ACTTGAAAAT TAAGAAATTA ACATCTTAA  TAAACGCAGC GGAGGTGGCT
721  GGTGTGACCG CCCGGGAGTA CGAGTGGGGA GCACCGTGCT GTGTTCGGGC TCTGGGATCA GCACGGGGCA GACAGAGGCT CTGTAAAGGC TGCGTGTGGG GCTTGGCACTG CCTTGGCCTT
801  CGGGTGCCAC TGCTCGAGTC CCCGGGTGCC CTCGAGCTGC AGGAATTT
```

Hits	Sequence	Length (bp)	Extent
89	1. Human DNA sequence from clone 431P23 on chromosome 6q27.	147,971	22/23
	2. Human genomic DNA, chromosome 6q27	331,211	22/23
	3. Drosophila virilis hook protein (hk) gene	4,054	20/20
	4. Caenorhabditis elegans cosmid T23B5	29,016	20/20

Table 5.2h: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 9	Cosmid clone(s): 7	Consensus length: 997

contig fragment assembly

Homology
Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

```
7   rev7,2,6,a      +---------------------->
6   fwd7,2,6,a                                          +
5   rev7,4,6,a      +----------------------<------------------------------>
4   fwd7,4,8,a      +-----------------<
3   fwd7,2,7,a      +----+                              >
2   7_33            +----+                              >
C   CONSENSUS       +----|----|----|----|----|
                    0   200  400  600  800
```

Consensus sequence

```
1    GCCCCGATGC yCCtCGCCCA CATGGATGkr GACAGAGGAA GAAGaGGCAg aGCCACAAAC TgaTtAAAca gTgaggbagg aTgCAaatac Agc...cag cA........ cctctAagG.
121  .....sac. cTG.GktA.. ...TACAaAg ...TGTTt. ...acGgC. A..mTTTTTT CCCCAaTcAA AaCCAAACA. TGAGctctAC AAggATTt.g CtTTttgTTg ggAAA.CATT T.ACTTCAGA
241  AgAgGTCTtT .ggGGATCAC AgAAAgAGTG CacaCaAAAG .GATCTTCC. .aCCTc.AGC CTCATcTACG TGCACTGCTG TAGACCACTC AgCTTTAgCT CTCCTCgATG GtgCacAGCC
361  TGTGTGACC. GTGA..CAGA TGGGAGACTA CAGCAGCATG CCCAGCTGGC A.T.CTgGag gaaTACGAgG gG.ACAAACA AGCAGCAGCC ATT.TACTCC AGCCT.GGCT GCCTCC.TGC
481  ATATATGCAC TGACCTGgCC AgTCTGTGGC A.T.CTgGag CCCAGGTAGG gaaTACGAgG gG.ACAAACA AGCAGCAGCC ATT.TACTCC AGCCT.GGCT GCCTCC.TGC
601  TCACTGTG.A G.ACCACCAG GGCTCGT.GG CTGGGG.tCA GcTCTTTCCT ACAGAGATGT T.CTCAATC. TGTT.CTTT. CCATGT.GCA CGT.CTTTcT .CCCT.CCCC TCCCCC.ATGT
721  A.AACAG.CA GAGCCA.Tc. ACCATCTGG. CAT.TTTCAG cT.GcTAAA. TAATT..CaT TGCCTTTTT. GTTAACACAG AGaG.AGTGT CtCTGG.CCC A.GGGAGCTG TTGG.ACCT.
841  TCAGCaAATA .AT.CAagGa G.ACTCTTAG C.AACGTCTG .AGTCCCTTC TgcCAC.Cac CA.CCACTCC Ag.CACCCAr sAGTtAGGGg TCCksCAGGA ATTCGAAATA AAGCTTGTGG
961  GATACCGTGG GCCGCGAGGG GGGGGCCGGG CGCCCAA
```

Hits	Sequence	Length (bp)	Extent
70	1. Oryza sativa subsp. japonica BAC clone 34K24	142,852	22/22
	2. Oryza sativa BAC clone 1.H19	77,605	22/22
	3. Oryza sativa subsp. indica BAC clone 16F19 php20725 region	70,311	22/22
	4. Sus scrofa mRNA for soluble angiotesin-binding protein	3,819	22/22

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Table 5.2i: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambiguous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.
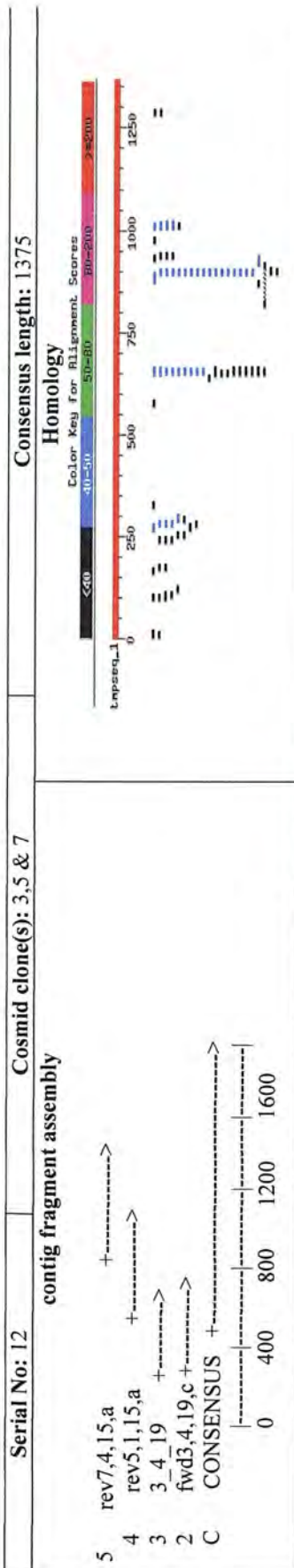
Serial No:10	Cosmid clone(s): 2, 7 & 12	Consensus length: 1995

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

tmpseq_1

0   250   500   750   1000   1250   1500   1750

contig fragment assembly

14	2_1_14	+--------->
13	2_1_23	+------>
12	fwd2,1,23,c	+-----> >
11	rev2,1,17,d	+---->
10	rev2,1,14,a	+--->
9	fwdorrev2,1,	+--->
8	fwd2,1,14,c	+--->
7	12_30_18r_rt	<---------
6	12_26f_rtt	<-----
5	11_27r_rtt	<----
4	12_29r_rtt	<----
3	12_28r_rtt	<-----
2	7_34	<-----
C	CONSENSUS	<------------ +

0   400   800   1200   1600

Consensus sequence

```
1     ACATCAGCTG CTGGGTCTTT GTCACCTCTC TGTCTTAATT GCTGATAACA AAATGAGTCT CTGAGCGGGT GGTATACTG
121   CTTTGTCTCC AGACTTTACT GACTCCGGGG TCTGATCACC GGGACAGGTT CTGGTCTTGG GCGGTGTTCT CCCTGTTGGC
241   TACATGTTCT CTGACCCAGC ATCTGTCCTG CTGGTGATTG GACAGGAGCC TTCTGCTGGG AAATTGCCTG TACCATGCCC
361   CCTTCAGTTT CACTGCTGAG AGCTGTCCTG GGGGCAGCGC GCTGGGGATA GCGTCCTCAA CACTCAGGGC AGGATGTGGC
481   GCTCTGTGAC ATCTkTkTTT GGGrAATCyC CAAATAGCGT TkTGacTkkC TktGacTkkC GttGGgCAAG AtCcaga.CT
601   ACAGTTtaTC ACTtCCTAAA AAatTcagGC CTCTTCTTGA AAGTCCCTTC GCTGTCTCTG CACTCTtGCA GttGGgCAAG
721   ACAGTTGTGC TCCAGGATGG ACTAGAGAAG AAAGCACAGG CATAGTTTTC TGAAAATCAA GCTGTCTCTG AGGTCTACT
841   AAATAAGTCA TCATAGGAAA ACTGTTCTGT CCAGAGCAG GCAAGAGCAG AACATGGTGA T.CTTATAGA AGGTGATTGT
961   AAACCCTCTT TAATTTAATC AGGCCCACAA ATGTATAAAT TCAAGAAATA AATGCTCACA ACAAACCTTT GTCCCTGCAT
1081  TCTTGTTACG CTGTACCTTT GATACAGGTT CTTATTACCT GATATGTAGC AGAAGACACA CTGCTAGGTT TGGATGATTG
1201  AAAACCAAAA CACTTCTCAT TTCTAAACTTG CCTTTGTAGG GTCTGCATGC TCTTCCCAGT AAGAGCTTTG ATCTACCTGG
1321  GAGGTCTCTG ATGCCTTCAG GTGTTTCAGT CACACTGGGG CGCAGCTGAA ccattCCTGC aGCaACCCCG GGCTTCTCAT
1441  aaCTA.GaTG TCaaaaagaT TCaGG.G.CG TTaATGTCAG TGTCTTTCGC TCaGTTTAaA GCaCCCTAaT TaACCagGCG
1561  GTGAgTgCAG TgCTGGCAGa CagCAGTaaT GGCaAaaaAa TCCCATTAAC TGGCCTCaaA TGGCTACaAG CaGtCCT.CC
1651  CCCaaTgag grCacaTGca aCaCCTaCCT GCaCAGGCaT CTTGCGcaTcT GTCGcaTcT GCTGg.CcTT CagGGaACGG
1801  ACCGGGCTTn AAAAATCTnT AGCCTnGnTn AAGGGATAAG AGCTGGTCaC CATGAACTGT CACACGCATC CGGCATATAC
1901  CACAAATAAG CTAAAATTAA CTAAGCCTCA GGGCATCGCT TTCATCTATT TTAAGGTTCT GCTGCCTGAr GATAA
```

Hits	Sequence	Length (bp)	Extent
12	1. Drosophila melanogaster genomic scaffold 14200001386035 section 44 of 105	218,434	20/20
	2. Human chromosome 14 BAC containing gene for type 2 iodothyronine deiodinase (DIO2) gene	154,796	20/20
	3. Human BAC clone GS1-587J1 from 7p12-p14	98,433	20/20
	4. Human DNA sequence from clone 403L10 on chromosome 6q25.1-26	60,215	23/24

Table 5.2j: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GC programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vecto. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with fo most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indica ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 11	Cosmid clone(s): 7	Consensus length: 858

contig fragment assembly

Homology

Consensus sequence

```
  1  GGAGGCGAGC ATCCTGGCAA GCCTGGTTAC AGAGTGTTCT CCAGAGTATA ACACGTTATG AATAGGGCTA CAGCTGTAAC CTGGAGAAGG TCCCAGCCTC TGAAGCCTTT GCCTTTAAAC
121  GGGGCTTTAC CAAACACAGA CAATTCCTCC CATGCCCGCG CCCCCCTCCA CACACAGCCA AACAGCAAAG AGCATGGCCA CTgTCCcAgA GCCCTGGAGG AGTGAAGCGg aAAaTGTAA.
241  CTCCTCATGG CCAGGAGAGG ATCCTTACCT GAGAAGAACT GGCTGATAga AgTGAGCGGA AGGCgAgCGG GTTTgCAAA. GGACATGGAG AGTGAAGCGg aAAaTGTAA.
361  GCCAtGCCTG CCA.CCAGGG .AGtCG.AgG CAAGCaCAGG gAGGTgTAag AGgCAgAACc ATGATGAAag TTtCCGCAaT gTTCCTgcTC CCAAaTGCAG TTCCCTgTgA AgAAACAgTC
481  gTggTCATgc CTCTGGGTgA CTCTggCACA CAgTCTACAG TGGgCAAGgC TGAGCCAGGG GACCTGCTTC CATCCAGCCA GTCCGCCACC ATTTCAGCTC CTTCgCCCAF
601  gTTTAAgCCC TTgCCCAgTT CCTCAGGGGA GCTCTGGATC ACGAGGATGT AGTTGCCCAG GGCATTAGCT gAGAGGAAGA GGAAGAGGgC CCCATgCagc AGAGCAGGag AGAAAAgTGC
721  ggTGgTgGAG GGgTCTCTGA ACATGCTGGG GATGAAGAGA AAGATCTGGA GGACGAAGgT CACTAGGgAG ATGCACAAGA
841  AGT.AGGyGG ggAGCgAACA ACATTgAGCA ACCTGAGATT CCTAGCATTG CGGATTAC
```

Hits	Sequence	Length (bp)	Extent
45	1. Human chromosome 14 clone RP11-463C8 containing POMT2 gene	180,331	151/186
	2. Human target of myb1 (chicken) homolog (TOM1), mRNA	2,310	22/22
	3. Human unknown mRNA (accession: AF040251)	899	22/22
	4. Human DNA sequence from clone CTA-286B10 on chromosome 22	87,869	22/22

Table 5.2k: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 12	Cosmid clone(s): 3,5 & 7	Consensus length: 1375

contig fragment assembly

```
5   rev7,4,15,a                    +-------->
4   rev5,1,15,a              +-------->
3   3_4_19          +------->
2   fwd3,4,19,c  +------->
C   CONSENSUS    +-------->
    |----|----|----|----|----|
    0   400  800  1200 1600
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

Consensus sequence

```
1     ATATCGAATT CCTGCAGCAC TTGGTGAGGT ACATCCTTTG CCCCCACAGG CGGTTGTTT CTATTTGTCA CACTTCACGT TTCTAAAATG AGAACTCTTT TTGCTCCTTGT TTTCCTCCAC
121   TGTTCCTCAA TTTCAAGCTA ATTTCACGGC TTTCATGGAT TCATGAAAT GAATAGATGG AAAATGAACAA TACACGCCAG CCAATACTTC AAGGAATGAA AAACCCAACA AAACACTCTG
241   CTTTCATTCA GAATTCATTA CACCTCTCCA CCATTTCCTT TCTTCTTTTT AATTAAGTGG TTAAAAGAAA CGCAGAAGCG TTGAATGTTT TTTGATGAGC CAAAGTCTTT CTTAGTAATA
351   CAGGtGGCCT CaAGTCATTA gtGACAG... ..ATGATA.C gGAaGCAATG AAAACGTGCA TTTAAATCAA AGGCCTCAGA TACACCATAG GGAACCAGCC TTGGGAGGAA CACCAGCCAC
451   CCCACCGAAC CACACCAGAC AGCCCCCCCC CCGAATATGC CCGGGAACC ATATCCCGGG GGAAATGATA ATATCCCGGG CAAAGGGGGG TGCCCAGAAA AGCCCCGTGG
601   TGAAAAAAAA AAAAAATATG GGTGATGCGG GAGGGTGGAA AATACATCC CCCAAAAACA ATAGAAAAAA CAAAGGGGGG TGCCTCCGTT TTTTTGGAGG CGGGTCAAT ATATGGGGGC
721   CCTTTTTTCC CTCCCAAAAA AACCTCCCCC GGGGGGAGGG ATTTCGGGGG GGCTCCCCCC TTTTTTTAT TGCCACTGTA AATGAGCAAA GAAATCCAAA
841   TAAACGGTAT TysGGAAGAA ACAAGCATT AATATTCAAA AACATCCATT ACCTCAAATT TACATCACTT TTTTCCCCAT CAAAACCAAA CATGAGAAAT
961   TCTGTTTCAA CACCAAATTA TATCTTATTT ATAGTGCGGG TGTTATCACA TAAATAAGGC ACACAAAAAA CCGCATTCGG AGAAAATTGT ATGCGGCCGT AGCGGACGCAA GTAGGGGCGG
1081  GTGGGGGGTA AACAGGACG GCATAGTTTG CAGCGGGACA GAGTGTATAT GACATCATTA CTGTCAGCGA CTTGATCCGCA AGCTCGCCCG CGTATATCAC GCAGCTGTAG
1201  CGCGCCGCTT TGTTTCTCAT CCAGAGAGGC ACGGACTTGT CCATCGTGT TCATCGTTTT ACACCTCATC TGTGACTAGT TTTCAAACAA GCAGCATCTA GGGACGCCAG
1321  CCGTTCGGTT GCCGGCGCAC GAGGAGACCT CCATTTCGCG GGTCGGGGCG CATCT
```

Hits	Sequence	Length (bp)	Extent
79	1. Drosophila melanogaster genomic scaffold 142000013386050 section 44 of 54	286,418	28/30
	2. Human PAC clone RP4-715F13 from 7q11.21-q11.23	145,878	21/21
	3. Human clone DJI015P16A	174,725	21/21
	4. Wolbachia endosymbiont of Trichogramma evanescens outer surface protein precursor (wsp) gene	558	20/20

Table 5.21: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambiguous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.
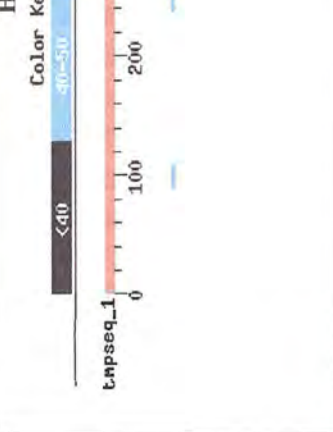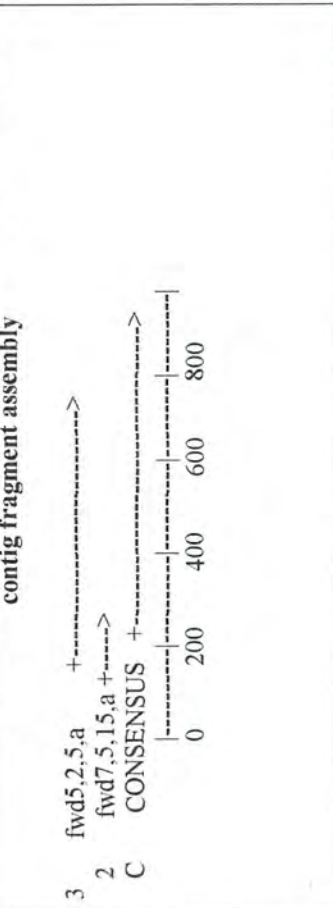
Serial No: 13	Cosmid clone(s): 5	Consensus length: 484

contig fragment assembly

Homology
Color Key for Alignment Scores: <40 | 40-50 | 50-80 | 80-200 | >=200

Consensus sequence

```
1   TGCCGCAGGG GAGGGCGGGC AGATaATCTC CTTAAGGGGT CTCCTTCTAG GGGGcAGGCG cCTCGcGGAA CTTATCTTAC tGTCgCCCAT GCtGGgtTGA
121 GtTTAGTaaA AGTTTcCAGA GTTcCCGcAG CTCtGtTAAT TCttCAGCmG cAAcAGcCAA GTTcATcCCG CCCCCccgGG GCCATAAagG GGTTtgcGsA AgGGGCtTCC
241 gGCTgGGGAA ATcCTcaAAG GCTTTTCTCC cTTTTACGcC CAAGCCCgCC CGCAgGGATG tTGAgGGGTC aGCTgATGCt CctTCCTTcA CCTtgGCCct TCCCATggCt
361 TCTTtaAATA AAatTCgCs AGCCCGcGa.A AAGtTGCCcg GCGtTgaAGC TtcCCAAagG GGCCTcgGGG tttTTGcCAt TACcgGGGG ATGGcCAGTt GAGgcCAGTT GCATGTATAG
481 GCAA
```

Hits	Sequence	Length (bp)	Extent
24	1. Human DNA sequence from PAC 179D3, between markers DXS6791 and DXS8038 on chromosome X	76,094	20/20
	2. Human PAC clone RP4-740D2 from 7p14-p15	141,990	19/19
	3. Human mRNA; cDNA DKFZp434H1322	5,333	19/19
	4. Human DNA sequence from cosmid LUCA3 on chromosome 3p21.3.	25,067	19/19

Table 5.2m: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 14 | **Cosmid clone(s): 7** | **Consensus length: 800**

contig fragment assembly

```
3   rev7,2,7,a       +------->
2   fwd7,4,4,a    +----------------->
C   CONSENSUS   +-----|-------|-------|-------|-------|
                 0    200     400     600     800
```

Homology

Color Key for Alignment Scores
| <40 | 40-50 | 50-80 | 80-200 | >=200 |

```
trpseq_1
         0    100   200   300   400   500   600   700
```

Consensus sequence

```
  1  AAGAGTGGTG AACACCCTCT TAGGACTCTG CCAGCTCCAG GTAACAACAA GCTGTCCTGC CTACACTGCC ACTGCTCCAT CATCTTGCAG GCAGGTATAC AACGACCAGC CCATCTCTCA
121  CCACACACAT CTTCCACCAA CCAACACCCT CCACACACCA AACAGATAAT CCCCCCACTC CTCTCCCAAC ACCTCCTACC TCAATAACCA CCAACCCAAT CTCCTACCAC
241  CACCTTCTCA CCTCTTCTCC ACCCCTTTTC TTCCCCCTTT CTCCTTCCCA CACACCCTAC TAACACCTTA CCCATCTTTA CCCTCTACCA
361  TTCTCTTCAA CCTAATAACT TCACCCTTTT AAAGCTCCTC ATCCCACCTC TCTCACCACA ACACCACATA ACACCACAAA TTAACCGAAA
451  TCATCCACCC ATACCATCTC CACCCATTCA AACTTTTACT CTATCTCCTC TAGACAmAAr AkAGACAGAG ACAAAACTTG ACAACTTTAA TTTTTAAGCA
601  AGCCTTGAAA AAAATTCCA CAAGAATTGT GTTGATGGCT CATGCAGATT TATATATTGA CAAATGGCAA GCACAGAATT AGAACATCTC TTTAAGAAAG
701  AATCTGACCA CCCTTCACG ACTCAAAATA TTTCTCCCCA ATCTACTTCT AGTCTTCACT TGCTTGATGC
```

Hits	Sequence	Length (bp)	Extent
25	1. Human chromosome 16 BAC clone CIT987SK-723D3	49,746	22/22
	2. Drosophila melanogaster genomic scaffold 142000013386043 section 6 of 8	274,591	21/21
	3. Human PTS gene for 6-pyruvoyltetrahydropterin synthase	85,084	24/25
	4. Arabidopsis thaliana chromosome II section 5 of 255	70,768	20/20

Table 5.2n: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for Cor G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 15	Cosmid clone(s): 1 & 7	Consensus length: 1022

contig fragment assembly

```
3   rev1,1,15,b      <-----------+
2   fwd7,5,12,a    +---------->
C   CONSENSUS    +---|---|---|---|---|
                 0   400 800 1200 1600
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

```
tmpseq_1
       |----|----|----|----|----|
       0    250  500  750  1000
```

Consensus sequence

```
  1  ATGATGTTGC AGGCAGGTAT ACAAGGTGCA GCCCTTCTGT GAGCAGAGAC ATGTTCCAGG AAGCAAGAGC TTGCAGACAG CCAAAGGCAC TATGAAAATA TAATAAGTTC AAGTGCTCTC
121  CCAACAGCTC CTAGGTCAAT AAGCAGGAAG GCAATGTCCT AACACCACCT TATCATCTCT CATCCACCTC TTTTCTACTC AATCCAAAAC AAATTCACCT TAAAACTCCA
241  AAAACCCAAA AAACCTAACC CATCCTCACC CCCAACCAAT CTCTTAAACC ACCCTATAAA TCTCATCCTC TTCAACATAA TCCCACCTCT
361  CACTCAACAA CAACACACCC ATCAACACCC ACATATTAAC CCAAATCAAT AACCTATTAA AAACTATTAA CTCTCACACA CTAAATAAAA ATTCCAACTT
481  CTTCAAAAAA AAAATCTCCT TTTTCCCCTA TTTTCTTTAAC TCTTAAATCT TTTCTTCACAT TTATAACTTT TTCTTCTAAA TAAATAACAT CTTCAAATCC
601  TTCTAAACAT CCTAATACAT AATCAACAAA ATTTATTCAT aTaTwAwTmT mTwGGkGTm TTmTGTmGhT mTAkTmTmG ThTwmTGGwk TGTTwdTmGw GGTwTGThTw
721  kGwAGTAwTw mGwGGAAGTG GTAwAwAGAw TTTwAwATGk GGwGAATTAA mTGGTTrGGA TdGTmmmmT TGGmTmkTmG rdGGGGGnmm TmrGmmTGGG TGmGmGmGGT
841  TGTmGTmGmI mGTGTGGGmG TTGGGGGGTm mGGGmmmmnm mkmGTGGGmG TAGGGTGArT GTWGmkGGwk kGmwGTmTGT GGGmAwGGmG GATGGGAAAnv mmTGwAdTmT
961  TTGmAnAmwG TmTTGGAGGm GwTmhmmTTm TTmmmmGnmT mmTTCTTmTT mImmTTmTTG Tm
```

Hits	Sequence	Length (bp)	Extent
13	1. Paracentrotus lividus mitochondrion	15,696	22/22
	2. Human DNA sequence from PAC 849L7 on chromosome Xq21	128,169	21/21
	3. Human Xp22 BAC GSHB-433024	207,957	23/24
	4. Drosophila melanogaster genomic scaffold 142000013386050 section 47 of 54	285,495	22/23

Table 5.2o: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 16	Cosmid clone(s): 5 & 7	Consensus length: 646

contig fragment assembly

```
3   fwd5,2,5,a    +------------------------------------->
2   fwd7,5,15,a   +----->
C   CONSENSUS     +--|----|----|----|----|--
                  0  200  400  600  800
```

Homology

Color Key for Alignment Scores

```
<40   40-50   50-80   80-200   >=200
```

```
tmpseq_1
         0    100   200   300   400   500   600
```

Consensus sequence

```
1    AGCTCCAGGT AACAACAAGC TGTTGCCTGC ATAAACTGCC ACTGATAAAT GATGTCAGCG GTTGTAGTAT TATAGGTGTT GGATGTATTT AGGAATGATG TTTATAGGTT AmGAGATAGG
121  wGCGAAAAG  CGCTATGTGA GTTCAAGGGG GAAAGCCGAA ATAGCCTGGC CTCCACAGCGC CAGATGCTCA CACCTCTACT CTTGAAGCCT CCTGTCATGT GCCATGGCGT GGAAGGTCAA
241  GCTTCCCAAG AATGTCTCCG GAAAGCCGAA ATAGCCTGGC AAGGGTCAC  TCGTTGCTAG CAAGCCAGAA AAGCCACGCA GGAGTGCGAA AATTACGAAA AAGACCTGGA
361  GGGGGGCCC  GATCATGGCC GCCCTCTAT  GAACCCTCTT TGCAACGGCC CAGGTGGTGG GCCTAACGAA CCACCCTAAA GGGCTGCCAT AATGGGGTTT
481  AGAGATGCAA CTTGCAATCT GTATAGTACC TAAAGTTGCC AAACCCACCA TACCGCCCCT AAACACACCC AGGCTCCAAG TGTAAGGCCA TCCAAAACCC
601  CCCCCAACG  AACAAAGGAC ACGCGAGGTC CTCTCAGCCC ACACCA
```

Hits	Sequence	Length (bp)	Extent
9	1. Drosophila melanogaster genomic scaffold 14200001338605 section 9 of 63	267,518	20/20
	2. Arabidopsis thaliana chromosome I BAC F24J13	87,400	20/20
	3. Drosophila melanogaster, chromosome 2L, region 27C-27C, BAC clone BACR13J07	142,141	20/20
	4. Mus musculus T cell receptor gamma locus, TCR gamma 1 and gamma 3 gene clusters	105,726	20/20

Table 5.2p: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 17	Cosmid clone(s): 3, 5 & 8	Consensus length: 1085

contig fragment assembly

```
5   rev3,1,1,a      +--------------->
4   rev5,1,6,a          +--------------->
3   rev5,1,7,a          +--------------->
2   fwd8,2,6,a  +------------>
C   CONSENSUS   +---|---|---|---|---|>
            0   400  800 1200 1600
                1081 CTCCC
```

Homology

Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

tmpseq_1

0   250   500   750   1000

Consensus sequence

```
1    AGGAGCTGCG AGCTTCCCAG GCCAGGTGTG CCAGGCTACA GCAGAACATT CAGCAGCTAG AAGCACAGTT GTGCdGGAGC ATCGAGCATC AGAGTTGGCT CTCAGAAAGG
121  TAAATAGTAA AACCTGTTGT AGAGCTGCTA rGTCTGGCTG CAAGTGATTC TTTTGkTCCT GCCAkTTCCT GGCAGATGCA CACGCkGkTT GTCAkAGTAT ACCTATGCTG TCCTCATCTC
241  TsTGATTyCA GCAGAAAGCA AGCyTGCGG GAswGGCTGG TkAGCATTCA GAGCTAkGGT TTTTTyAGkd wywGCCACCA GCCrCwGAGG GCCwGGCkGG GGACArkACG TCyGkkCTCA
361  yCTGCCTAC AACCkgsTGC kCCACCGGGG kyCGGGCCAA CAAGCTGGkG GdCmndCACA wCATAGkkTG TCACbrTGGC AChACGAyTT GTGCCCAGkA CrCTkGGGCs
481  CACCTAAGCd CCTcKCCrCs AACmmmCmAG CGTTGGKCmC sGCyyCkGyA aaaaaGAcA. .GGaaTTATG ATAGCAGAtG CAATGA.AAA CGTGCATTTA AATCAAAGGC
601  CTCAGATACA CCATAGGGAA GCCTTGg GTgGTTCTCA TGCCTCCCAT CCGTTCCTCT AAGATATGAA GCACCACAGT TTCcccCAGG CGCAGTTTCC TCTTTCAGG
721  CTCTGGGAAA .tCACCtC.. .sg.tTTtha bvT..AaAaC cCmacbtgtt AsrcaggsAa aagcwctt.. tAc.cAaaA. ...yAgsckt gGGgTwt.g gtagttadaT yAcacgCCch
841  acghchg..c vgAdAvT..y AArAAAAdG. ......tctt ccTtTTtdtc cgAawmngvg dgdaaaAcrg GtccCTcTtT thbccAccaA .......aa AAAAaCaC.. ...cAhcar
961  wat.CmgCCA dddbaCmycm cCbcCaGG.. .GcgcggCA aattckgrgs rCccCgtavt gdgaGCCcac ..kggAyaac agcAkTgctd ccttavaGGT ahCcgrGCys ykyTGtGww
1081 CTCCC
```

Hits	Sequence	Length (bp)	Extent
35	1. Human DNA sequence from clone RP5-1108D11 on chromosome 20q12-13.11.	152,209	23/24
	2. Human KIAA0575 gene product (KIAA0575), mRNA	5,285	19/19
	3. Drosophila melanogaster genomic scaffold 142000013386047 section 11 of 52	272,650	19/19
	4. Mus musculus casein kinase II alpha 2 (Csnk2a2) gene	1,498	19/19

Table 5.2q: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

157

Serial No: 18	Cosmid clone(s): 1	Consensus length: 152

contig fragment assembly

```
4   rev1,2,9,b   +------------>
3   rev1,2,7,a   +------------>
2   rev1,2,1,a   +------------>
C   CONSENSUS    +------------>
            |-------|-------|-------|-------|-------|
            0      100     200     300     400
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

Consensus sequence

```
1    GGGGAACACT CACAGGCGGG CACGAAGGGC TGCATGTCCT GCACAGGGGA TGGAAACAGG GCCAGTCCTC AcCAcACTCC ACAA.AGGCG ATTTTTgAaT TCCtTCCCTT TTTAAGTCAT
121  TGCTCCCCAA GGACTGCCAg TGcCTGkATT Aw
```

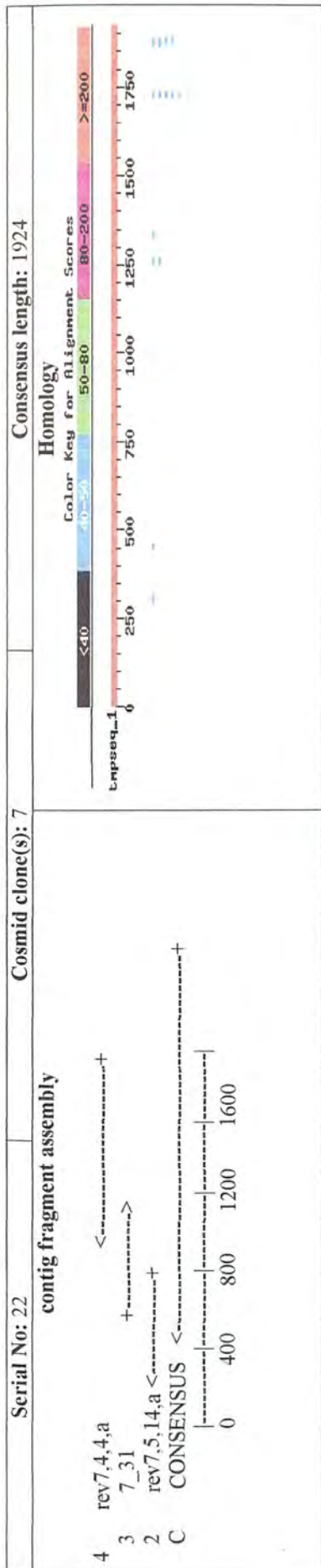| Hits	Sequence	Length (bp)	Extent
27	1. Human DNA sequence from clone 55110 on chromosome 1q32.2-41	90,429	20/20
	2. Human 3q25-26 BAC CTB-177N7	82,263	19/19
	3. Human BAC clone RP11-507E21 from Y	107,402	19/19
	4. Human 12p11-37.2-54.4 BAC RPCI11-49G2	156,314	22/23

Table 5.2r: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for Cor G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 19	Cosmid clone(s): 3	Consensus length: 758

contig fragment assembly

```
3   3_3_8   +--------------------------->
2   rev3,3,6,a  +------------------------------->
C   CONSENSUS  +----------------------------------->
    |-----------|-----------|-----------|----|
    0   200   400   600   800
```

Homology

Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

Consensus sequence

```
1    GmGgTGATCC CC.GGGCTGC AGCCACTGAA CT.CTGTCCA ACCAATCCCt AAGTT.TAAA TAATTCTTAA ATGTTGCAGA TTTTAAGCAA CTTTCTATGC TAGTAGACTG TTAGCCTGAA
121  AGTTCCAGCA GRACTCTTGC TCCATCTtCA GGAAATACtA GGCAAGTCTG ATTGCACCTC TGCAAGGAG GTTATGGGAT GTTATGGGAT GAAGCAGCTA GTATTCCTTC
241  TTGTGGCTCC AT.TAGACtA GGGTTATtTT TTTTTTTTTAG TTCAGATGTG TT..t.TTTT TTtTATA.TG CTTATAGACT GACAACAGCT .GTTTGCTGG AAGAAATGCA GTTTTTTAAG
361  GTTTTCTTTT GAATTTGAGT GTCTCACTCA GCTGTTGGAT TTTGTTGAAT TATTAAACAG CTGTGTATTA AATGCTATTG GCCAAGAAG AAGACAAAA TTGTCCACGT
481  CCCAAAACTT AACATTGTAT TGGGTTGTGA GCTCAAAATT TGCAATGTGA GAACATCTGT ATGGCAAACA GGTATACAGA TTGTAAGGTA AATAGGTATA CAGGTATAGG
601  ACCRGAGAGA TTATCGCGTT TACAAAGGAG TAATGAAATA AAGAGCTTTC CCATGTCCTG GGCTTGCAGA AGGGCTCCAG AGGAGTGATC TCCAGATAGA GATCCTTCCT CGTTGGGAGT
721  CAGCGTTAAA .TGGGGTCTA GGAGGGGTGA AGC.AGGC
```

Hits	Sequence	Length (bp)	Extent
42	1. Gallus domesticus DNA microsatellite marker MCW158	212	78/83
	2. G.gallus microsatellite DNA (clone 63C12	190	53/61
	3. Human clone RP5-1075C10	171,457	20/20
	4. Human syntaxin binding protein 3 (STXBP3) mRNA	2,508	23/24

Table 5.2s: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 20	Cosmid clone(s): 7 & 8	Consensus length: 922

contig fragment assembly

```
3   fwd8,3,6,a     +--------->
2   rev7,1,23,a  +----------->
C   CONSENSUS    +------|------|------|------|----->
                 0    200    400    600    800
```

Homology

Color Key for Alignment Scores
<40   40-50   50-80   80-200   >=200

Consensus sequence

```
1    AGAGCGCTCT CACAGATCGC GAGAGCCGC CCCGCTGGTG AGTCTGTGTG TGATGCACTG TACACACCAT ATGTGTGACA CACACATGTA CATGTCTTCG CGTACACGCG CTCATGATGC
121  GCTAGAGCTG GAGATGTGCG GTGCTCTCT TTGCGTCTCT ATCTCGTGTG CGTGTGAGAG AACGTGTGTT GTGCTCTCTC TAATGCTCTC ACATATATAC TGATATCCCG CGCCACACAC
241  ACATGAGAGT GAGAGAGTGT CTCCTTCTCT GCTATATATG CGCCTCTCCA CGCGCTCGCT ATATACGCTC TTCGCCTCTG CACATATCTG CTATGTGAAT ATGCGCGGTC
361  TGTGAGCCCC AAACTAGACC TCCAAAAAA CACAACTTAG GCATATTTCC CACCAATATA AAATAAAACA ACTTTAGCCC AATTTACTAT GCTTAATCAA GACAACACCG
481  GTATTTAAAC AAGTTTCTTT TCCCCAAAAA TACCACACTA GTCAAAAAAA AATTTAATTT TATTTGGGGG AAACGTATGA AGAAAAAAAA CCAAAAAAAA AAGGCCCCT
601  GTsGGGGGGC GCCgrmACGC CGTGCCCCCG GGGCTGAAAC AACACCACAG AGAGCCGCCAG AGCTCTGCAC ACAAGGGCCA GCACAAACAG
721  CAGAAGCTTC TTAAACGGGA CGGAAAGGAG ATGAGGGAAA AAAAAGTAAG TGGGGTTTGG GGAAAGCGGA CAAAGATGA AATGAAGTTG GTGGGTTTGG GGTAAAGGGT ATAGGATAGG
841  GGTTGTATGA TCAACTTAGA AACAAGGCTC CAGCTCCCGC ACAAGCAGTG GGGCCCGCTG TATAAAAGTG GAGAATCCCC TT
```

Hits	Sequence	Length (bp)	Extent
23	1. Human chromosome 17, Neurofibromatosis 1 locus	297,898	22/22
	2. Mus musculus chromosome 10, clone RP21-247L16	130,427	23/24
	3. Mus musculus clone UWGC:mbac37 from 14D1-D2 (T-Cell Receptor Alpha Locus)	132,090	23/24
	4. Human DNA sequence from clone RP11-503P21 on chromosome 20	93,365	20/20

Table 5.2t: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 21	Cosmid clone(s): 7 & 14	Consensus length: 1031

contig fragment assembly


```
9   14_32r_rtt        +--------->
8   14_33nr_rtt              +--------->
7   14_34r_rtt                   +--------->
6   14_33r_rtt                   +--------->
5   14_35r_rtt                   +--------->
4   14_31f_rtt                   +--------->
3   14_33dr_rtt                        +------------>
2   rev7,2,16,a +-------->
C   CONSENSUS +----------------------------------------->
        |----|----|----|----|----|----|----|----|
        0   400  800  1200  1600
```

Homology

Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

Consensus sequence

```
1    GAACGCGACA GGAAGCAACA AAGCAGCCGG AGGCCGCGGA CCCAGGGCCC GCACCCCACA CACAGAGTAC CCCCCCGGG CCAGACAAA AAAGGCTGAT CAACAAGGCG CATGAGCATG
121  GAGATGAGAA ACGCATTTGC GGGTCTTTCT CCCTCGTATG CGCATGCCCG GACGGCCCGC AGTGCGGGGC TGGCAGTCTG GGAGTAGGAG AGCGGCTAACT
241  GCTCTTCGTG GCTTGATGCG ACCCCATTGA GGAGGAAGTT AGTGGGGAGG ACTGGGGCG GTTAGAGCCG CAGGGGGCTC GATTGCCTTA CCGAGGCACA AaaAGTrwAC
361  TGAGTTAGTr ACKhADnTAT CCAmCTAvcT GGATCCCCnb GGCTGCAGCT CTTCCTTCTG GCAGACACGC TTCACTCGAC AGCTGGCAGC GTACCCGAT TCTTCAGCGT CCGTCGTCGC
481  TGCTTCAGCC TCGGCACCTC CTCCTTGGAA AgGCCCCGCA GTGGTGGTTG AgCTCCCGCA CCGAGACGCC GCAGGGGCGT GTTCTTCCC AGCTCCGCT
601  TCACCTGCCG GGTGaGGAG CAGGTGAGAA CCCAGTgcca acCCCCCAGT CAAATGCTAA GCACCAGTGC TAGAcTCCT CTGTGCTAga cCCCACAAAT gccAaccCt
721  AACGCcagwc CCCtCTtGtG GacctcCCCA aCACCaatcC CCAGTGACCC GAATGGACCC CCCCCCCCA AACGCCAACC CAATTGCCGG ACCCCAAGGC GGATCCCCCA
841  CGCCACAGCC CCAGTGCAAA CTCCCCACGC TGGAGCTCCC TGAGGCTGGA CCTTCCCAGT GTTATTCCTC CATGCTGGAC GTCCCCCATA CTGGAGTCCC CACTGCCAGC
961  TACCCACGCT GGACCTCCTC AATGCTGGCC TTCTCCCTGT GCCAACCCCC AGCGGCTGGAC TCCCTdATCy T
```

Hits	Sequence	Length (bp)	Extent
41	1. Chicken mRNA for nuclear b-Zip protein MafF	1,800	203/206
	2. Mus musculus gene for mafF	2,699	171/207
	3. Mus musculus avian musculoaponeurotic fibrosarcoma virus (v-maf)AS42 oncogene, protein F (Maff), mRNA	471	155/186
	4. Human v-maf musculoaponeurotic fibrosarcoma (avian)oncogene family, protein F (MAFF), mRNA	2,357	164/201

Table 5.2u: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

| Serial No: 22 | Cosmid clone(s): 7 | Consensus length: 1924 |

contig fragment assembly

```
4   rev7,4,4,a        <----------->
                         +-------+
3   7_31
2   rev7,5,14,a  <-----------+
C   CONSENSUS     <---|-----|-----|-----|-----|
                  0   400   800  1200  1600
```

Homology

Color Key for Alignment Scores

| <40 | 40-50 | 50-80 | 80-200 | >=200 |

```
tmpseq_1
          0    250   500   750   1000  1250  1500  1750
```

Consensus sequence

```
1     AAAAAGCCCG AAATCCCCCC CCCCCCCCCC CAAAACGCTTC CCCCCCTCCC ATTCAATTTT CCATTCAATA TTCTTGCCCTC TTCATTCATC CCTCCTTTCT TCACTCCCCC ACACCGGCCC
121   CATTTATCCG TCAAACACGC ATGGAAGATG TTTCACATGT CCACACCGAC CCTGTAGGGA GAAAGCCTCAA AGGGTAGGTA CCCTGGAAAC AACAGTGCCT GGGGGCCCTG GAGCATGCTC
241   TGGAGCTTTG GGTGCGTGTG TACCCACGGG GAAAGGGCCA AGTTGGTGTC CATGCACTGA GAAAAGGGT CTCAAGGATT CGTCAGAAGC TGCTGGTAGC ACATTGCCTT
361   CCTGCTTATT GACCTAGGAG CTGTTGGGAG AGCACTGGAG CTTATTATAT TTTCATAGTG CCTTTGCCT CTTGCTTCCT CGGACACTTC TCTCCCCCCC CAGGGGCTAG
481   TCCTTGTATA CCTGCCTGCC CGATCATGGA GCAGTGGCAG TGTmkGCAGG TAGGCATGGC CTGGCAGGCA GGCAGCCCCT CACCTCCCGC GTCCCCCCGG TGCATTGCCA CACAGCCAGC
601   GGGrGCAGGG GGGCTGrGGr GCCCAGAGCC TCAGAAAAAA CCCyAGGACC GGGGGGGGAT ACGTCCCTTC GGCGCCGGCT GGAAGGAAAA AAATTGGGCC
721   AAAAGGTTGG ATTTGAAAAA AAAAAAGArA AATTGCCCAT TTCCCTTACA GCAAAGGGGG TGGGGCCTTC CCGGGTGGG GGGCCGAAATC CCCCCCCAA CCAAAAGAGA
841   AAAAGGGGCA CCACAAACCA GGATCCGACC TTTTACCCAA CyCACCTTGG GAAATTGGGG GCCGAAATC CCCCCCCGGG CCCCCCAA CCAAAAGAGA
961   AAAAAAAACA GCCCCCAGCT TATGAGGGGG AAATCCTCTC TyTCtTTTTA ACArAAArAA TyTCtTTTTA CtACTAAGCT TACGAAAAAA AAAAGTCCAT GGACCTAATA TTTTAGGCAA
1081  TATTAGAAGG CAAGCGCTCA CACAACATCT ACGTCCCGCG TGTCTTCCCA CCCTACACTG CAACTGCCC TAAGGGCCAA AAAGGCCCAT AAGGTCTGAC AGGTGTGGGG
1201  CCCAGACGGG GTTCCAGGTA CCACCAGGAG GTTTTCTACA GTGCGGGGGA CAGGTACATT TTTATGTTAT GTCAGGGAG AAGAGAAGA GCAAATACCG AGAGTTGAT AGAGTTAGAT
1321  ACGAAGGTAA ATGTTTTTCT CTCTTTAGGA TGTGTCACGG AAGTTCTAGT TTTCCCTGTG AGAGGAATAA TAACTCGGAG GGGGCGCAGA GCAAATACCG AGCATCTTGT TTCCACTGAC
1441  TTCAGTCCTC CCAGAGTTGT GTGCCOGGCG CCTCACATCA ATCATCCCAT TGCTTTTATG TATTCCACCA CGCTCCCAGC CGAGTGGCG TATCTCAGCG ACTGAGCAGT CTAGGACTTG
1561  TGTATGACCA TAGTGGTTCC TGTGCTACAA GGAGGCCGGG TAGTAATGCG AGAGAAGTTA CAGCGGGCAA GCTCCCCAAA CTTGTCGATT CGCAACTTCA GATGATGTCC ATAAGAGGGT
1681  AACTTGGGGT GTGACCATAG AAAGGTTCTT CAGCCCGTCA CCTTGGAGAAG GTGCTGTACA GCTCTGGATA TTCCCAATTG CTGACCTTC ATATTTCAAT CCCTCCCACA
1801  TTGGGGGGTC ACAAGATGAT ATAAATGGGT GCTGGAGAAT AGCACAAACC CCACTGGTGC GACTTGACCA TGGGGTGCT TCCGGCAGTG AGTGTGGCTG
1921  TAAT
```

Hits	Sequence	Length (bp)	Extent
14	1. Human chromosome 14 clone RP11-463C8 containing POMT2 gene	180,331	23/24
	2. Human chromosome 14 clone RP11-493G17 and CTD-2516D11 map 14q24.3	210,204	23/24
	3. Human Chromosome 16 BAC clone CIT987SK-A-363E6	220,633	20/20
	4. Human Xp22 BAC GS-551O19	151,750	20/20

Table 5.2v: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambiguous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 23	Cosmid clone(s):7	Consensus length: 1575

contig fragment assembly

```
3   rev7,2,9,a      +---------------->
2   rev7,5,15,a  <------------+
C   CONSENSUS    <---------------+
             |----|----|----|----|----|
             0   400  800  1200 1600
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

```
tnpseq_1
         |----|----|----|----|----|----|----|
         0   250  500  750  1000 1250 1500
```

Consensus sequence

```
   1  ATTGGTGCGC GCTTGGCGTT TCCCTCCCTC GCTCGTGTAT ATATCCATTT GCTCCCCTG ACATAGACTA AGCAGCAGCC AGTCCTGATG TATAGACACG TGAGGTTTCT CAGGCGGTGC
 121  AGCGACTGTT CATGTGGATG ATAATGCCGT GGTAGACTAT GGTAGACTAT AGAGAGAGAG CAAATAAGAA AGACTTACCA CCAGAGTCAT GGATGATAGC TCCGGGAGAG GAAAGACACG TGAGGGGAGC
 241  AAACATGGCA CATGTCTGCG CTCTGCCCAG GCTGGCCTGC CTCAACAGCC CTGACTCAGA ATCCTACCAG CGCTCACCAC CCCTAGGCGG CGGCATCACA GCTTACAGAG
 361  CTCCCAGAC CTGAGTAGGT CCACAGTGGG GCCTGTGCTA CAAGGATGCC TTGGAGTAAG GCTAGAGAAG TCACAGCCTG GCAATCGCCC CAAATCTTGT CTGATTCTGC AACCTACAGA
 481  GTGACTGTTC CAGTTCCTTT GGTAACTTGA GCTATGACCA TAGAAAGGTT CTTCAGCCCG AGGGTGATGT ACAGCTCTGT ATATGGTTTC CCCTTCCCAA TTGCTGGCCC
 601  TTCCTCTTCC ATCCCTCCCA CCATGGGGGC TCACCAGATG ATATCACTGG GTGCTGGCAG AAGGGACTCA GACGTTTCTA ATAGTCTCCT TTATTATTTG GTAAAAGTGC AAGATGTGGC
 721  TGGGAGAAAA AAAATCTGTC TGTGTTAAGA CCATGGGGGC AGAGGTGAAA AAATTATTTA AAGGGAAATG GTGATGGGTG TAGTGTTTAG ATGGGAGGA AAAGGAGAAA GAGGTGGAGA
 841  TGGGAAAAGG AAATTGCGAA GATGTGTATC AAAAATAAGT TATATGTGTG AACTTTATGC AAAAATGAAG CCAAATAGCG TACATTTATA ATACTTCTTT
 961  TATCGGCTGT TATCACCAAC TGGGGCTGAC GTATATGCGA GAATGATGAT ATATGGTTGA AGATGGAGAA ATAAGCTAAG TTAGGGAACT GAGTAACCTG CGTCTAGCTG
1081  TGTTATTGGC TGTTCGGTAG GAAAATATGT CTCAATTTTT CGAGTTATTA TGGATCGTCG TTATCTGGCT GTCGTGAACT CGCTAGATT ATTTCCCCA TATGTGTTTT
1201  TTGCTAAACA GCGTGGTCAC GTTTCTAGGG ATGTGTGGGC GTATTCTCCA TTATCTGGGC TTAGTCATAC TGTCTGAACT CGCTAGATT ATTTCCCCA GCAAATTAAC
1321  ACAGTATCAC GCGCAGTCGC ACTCTGCACT AGGCTGCGTT TTTTCTTTCT CACACAAACA ATCACTTCAC ANTTATATAT AGTTGCTACA ATTTGGTTGC GCTGGTTTGT GGTGTCTGTG
1441  CTGCCGGTTG GTGGCCTTGG TCGTTGCTTT GCGGTGCCCC TCTCCGCTCG GGTTGTTGGT GTCGCCTCTC TTTTTTTTG TTCGGTTCCC CCCCTCCCGG TGCGCTTGT
1561  CGGGGGGGC GCGCC
```

Hits	Sequence	Length (bp)	Extent
20	1. Arabidopsis thaliana DNA chromosome 3, BAC clone T12C14	77,720	24/24
	2. Drosophila melanogaster genomic scaffold 142000013386047 section 37 of 52	261,694	22/22
	3. Cynodon dactylon chitinase (Cht2) gene	1,788	21/21
	4. Cynodon dactylon chitinase (Cht1) gene	2,103	21/21

Table 5.2w: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

Serial No: 24	Cosmid clone(s): 8	Consensus length: 452

contig fragment assembly

```
4   fwd8.2.5.a                          +------------------------------>
3   fwd8.2.5.b            +---------------------------------->
2   rev8.2.5.b   <----------------------------------------+
C   CONSENSUS    <--------|---------|---------|---------|------|------+
                 |---------|---------|---------|---------|
                 0        100       200       300       400
```

Homology

Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

Consensus sequence

```
1    AACsTCCAGG GGArAGCCCC AGAGCAwCGA TCGGTwGrCA wTAACGACkk TyTTAGGGAT CGGGCGTAwT TACGATTkCT TGAATAGGGT kCAATGTkGG wATCCGGGCy CCCCyCATG
121  AGGTCkTAyT GAwTCGArTA GCTGGAcAtC GAwTTyCytC AggAGgGgTC GTtTGTCt. CAtgCTCAct GCACTGAATG GTTCTGATGG AGATGAGGGC CTGCtTTCCC
241  CATTCCCTCC GCTCCGTGGA GgTCTGTGAA TTGAGACACA TGAGCATTT AtGTGCAGTT CCAgTGtATA ACAAGTGtgt TTCACTgCTC CCAGCTgAGg ATCTcTCTTG
361  CAGGcagCTC TCTGAGCTGT GTTCCCCAGA CAGTCACTCC AAGCCTACCC TGAaGCCACA GGGAtGATCT TGGCTCCTCT TGAGaTaGGG TA
```

Hits	Sequence	Length (bp)	Extent
28	1. Human DNA from chromosome 19, cosmid F21856	39,631	24/25
	2. Human 3 BAC RP11-7715	168,812	20/20
	3. Caenorhabditis elegans cosmid C09F12	16,919	20/20
	4. Barley yellow mosaic virus RNA for polyprotein RNA1	7,648	26/28

Table 5.2x: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

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Serial No: 25	Cosmid clone(s): 8	Consensus length: 850

contig fragment assembly

```
3   fwd8.2.18.a      +-------------------->
2   rev8.2.6.a  +----------->
C   CONSENSUS  +--------------------------->
           |-----|-----|-----|-----|-----|
           0    200   400   600   800
```

Homology
Color Key for Alignment Scores

<40	40-50	50-80	80-200	>=200

```
trkpseq_1
           0   100  200  300  400  500  600  700  800
```

Consensus sequence

```
  1  AGATATATGA TCATGCGGCG GAGCTATGAA CTGTCTCTCA TCCTAGATAG TGCTCCCATC TATATCATAT AATCATAGGG ATATATGCGG ATTCCATTAT CTCGATCCAC CGGCCGATCA
121  ATTATCCTGG GGATCATGGC CCTAAGCGCA GCATCATTCT CCTGTGTCAC CTCGCCTGCG CCCTCCGCAA TCGTAGCCTC TCCACTCCTC AACTCCCACT
241  CACCCACTCA ACATACTAAT TTTTATCTTT TATAAATTAAA CTTCAGTCCT CCCGTCTCC CCTCwGGGTC CCAAAGCCTC ATCACGCTCG TCCGCCTTC TTGGTTTTCT
361  TCTTCTCCTC TTTCAGGCCA GTGTCGTGGG CTTTCCCTTT TGCTCGCCGT GCACAGAAAC ACAACCCGAG ACCCACATTs ATsTsTkTGs sGAsAsAGsA ATATGTTGTG
481  sATGGTGATA GGGAGTTAAs TTGTsTTAsA GsTTTTTATT TATTGAATsA TTTTTTGGGG AGAGTAATTG GGGATTGATT sAsTTTTATT TsTTsTsATT TATTTTTAAT TssTTsATsC
601  CTGCssCsCt ssTTTGsTsT sGGAAGsAsA TsATAATAAs ATATTTTTTT GGTTGGTCTA GATTAGGGAT AGhgACAmGy GATTTTTTTG mAAAAGAmAG GTTTTTAGTT TTGwTAAGAG
721  GGsrTATTTT sATGTATAmT GTTsGsmTTA TmTATAAATG AGAyTTTTTT TGTTnmAmTT AAmGAmGAGG mTGhGTTTAC GTCTATTTGG AGTTGGTTAT TGGCmACTTC GGCTTTGCTw
841  CmGCCGCGTT
```

Hits	Sequence	Length (bp)	Extent
38	1. Human DNA sequence from clone 790B6 on chromosome 20p11.22-12.2.	106,179	25/26
	2. Drosophila melanogaster genomic scaffold 142000013386035 section 61 of 105	260,278	21/21
	3. Human chromosome 17, clone hRPK.293_K_20	185,254	21/21
	4. Danio rerio pes mRNA	2,214	33/37

Table 5.2y: The consensus sequence of the cosmid subclone. This consensus was created by the fragment assembly system (FAS) of the GCG programme obtained originally from the sequences of the subclones in the tertiary screening of the chicken genomic library in the cosmid vector. A schematic display of the consensus with the contigs created by 'GelView' function of the FAS and the comparison for homology with four most homologous sequences also are shown. In the sequence uppercase letters indicate a definite nucleotide and lowercase letters indicate ambuigous nucleotide between the fragments. M is for A or C, R is for A or G, W is for A or T, S is for C or G, Y is for C or T, V is for A or C or G, H is for A or C or T, D is for A or G or T, B is for C or G or T and X/N is for A or C or G or T.

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hybridization signals, but after cloning and sequencing none was found to correspond to a chicken leptin-like DNA sequence.

The failure to identify leptin-like sequence in any of the three DNA libraries might be related to the structure of the leptin probe used for screening or to the screening methodology.

Two features of the nucleic acid probe used for these experiments are critical to the successful screening of recombinant DNA libraries (Ausubel 1998). First, the probe must hybridize only to the desired clones and not to any other clones. Thus, the nucleic acid sequence used for a probe must not contain any reiterated sequences or sequences that will hybridize to the vector. A general sequence comparison with the UWGCG programme was carried out before using this probe and it showed that it did not contain such sequences. Second, the specific activity of the probe must be at least 10^7cpm/µg. This criterion was scrupulously adhered to in all experiments and hence the absence of a signal could not result from a low specific activity of the mouse leptin probe. False positive signals obtained when screening cosmid library screening seems to be common problem (Ausubel 1998). In fact, most of the sequences identified from the tertiary phase of screening the cosmid library had reasonable homology with the mouse leptin probe used (see table 5-1a-o, column marked for the similarity with the probe). This could explain the consistent hybridization noticed during all the three phases of the screening.

The failure to obtain hybridization of the probe to the chicken adipose tissue cDNA library may be because the leptin gene was not represented. Since leptin acts as a satiety signal (as known in mammals) the expression of the gene in adipocytes would be dependent on the bird being fully fed at the time of death. After the library was screened and no hybridization was observed, an enquiry to the technical support department of the supplier (Stratagene) revealed that the adipose tissue cDNA library was obtained from fasted birds. This could explain the absence of any positive signals from the cDNA library screening.

The failure to identify a mammalian-like leptin sequence from this library could be due to a different intron-exon structure in the chicken leptin gene, which is different from that in the mouse leptin gene, preventing a strong hybridization with the mouse leptin probe. If this were the case, poor hybridization would have reduced the sensitivity of the probe relative to background hybridization. It is also possible that the leptin gene sequence was under represented or unrepresented in the library due to a failure of it to be ligated into the library, or due to instability of the insert containing the leptin sequence in the vector. However, the most likely reason for the failure to clone chicken leptin gene from the gDNA lambda library is a low homology of the chicken leptin gene with the mammalian leptin gene sequences or a complete absence of a leptin homologue in the chicken genome.

The most promising results were obtained with the cosmid gDNA library. However, none of the clones identified from the hybridization signals showed a sufficient sequence homology with mammalian leptin sequences. Many of the sequenced clones revealed short stretches of homology with the mammalian leptin sequences (table 5.1a-o), which probably explain the consistent hybridization signals obtained throughout the screening procedure. This also confirms that the sensitivity of the probing technique used was adequate.

Therefore, failure to identify a chicken leptin-like sequence from the chicken cosmid library could be due to an omission of the leptin gene from the entire library, a low homology of leptin between mammals and birds or complete absence of a mammalian-like leptin sequence in chicken genome. A known disadvantage of cosmid vectors is the instability of many cloned eukaryotic sequences due to recombination and deletion of portions of the insert DNA in the prokaryotic host cell and very large segments of eukaryotic DNA containing unstable constructs of repetitive DNA (Dame 1998). Considerable data exist on the nonrandom representation of sequences of both prokaryotic (Lucier et al. 1994) and eukaryotic origin (Huber et al. 1993) in gene banks constructed using various cosmid vectors, hosts, and packaging mixtures. Omission of the leptin gene sequence from both the chicken genomic libraries in lambda and cosmid

vectors seems improbable. Therefore, poor homology of the chicken leptin nucleotide sequence with the mouse leptin probe or complete absence of it from the chicken genome seems the most likely explanation for the failure to identify chicken leptin gene from the screening of the chicken cosmid DNA library.

Overall, the data from screening the chicken cDNA and gDNA libraries with the mouse leptin probe suggest that chicken leptin sequence is unlikely to be homologous to that of mammals, if not totally absent in the chicken genome. The positive signals obtained during the screening of the genomic libraries, both in phage and cosmid vectors, are most likely to be due to short homologous sequences hybridizing with the probe. It is possible that the homology is so low that it is close to background hybridization of unrelated sequences.

Several laboratories in Japan and the USA have been in contact with the Roslin laboratory since the publication of the main findings of this thesis to say they too have been unable to clone non-mammalian leptin from chicken DNA libraries. These unpublished observations support the view that failure to clone the leptin gene by screening DNA libraries was not due to poor methodology. Rather, it seems more likely that the chicken leptin gene, if it exists, has such poor homology with the mammalian leptin gene that the DNA library screening approach does not have the resolution necessary to clone the gene.

CHAPTER 6

PHYLOGENETIC ANALYSIS OF LEPTIN-LIKE SEQUENCES

6.1 Introduction

Following the failure to clone the chicken leptin gene by RT-PCR (chapter 3) or by screening cDNA and gDNA libraries (chapter 5), the only evidence for a leptin-like sequence in the chicken genome is that provided by the Southern analysis (chapter 4). The latter suggests that if a leptin-like sequence is present in chicken genome, it is less than 75% identical to mouse leptin. This fails to confirm the 95% homology between chicken and mouse leptin genes reported by Taouis *et al.* (1998) and Ashwell *et al.* (1999a). The very high percentage (95%) of identical nucleotides between the putative chicken leptin and the mouse leptin sequences is unprecedented and is not observed for the same comparison between other cytokines (Table 6.1). It is contended that the published chicken leptin DNA sequences are of mouse origin. In order to put these results into perspective with the present concepts of molecular evolution, two approaches were used. Phylogenetic trees based on leptin and prolactin (being similar to leptin as a circulating hormone and a cytokine) sequences were compared and a statistical analysis using synonymous substitution values between chicken and mouse leptin and twenty randomly chosen gene sequences of chicken and mouse was carried out.

6.2 Molecular Phylogeny

According to the evolutionary geneticist Theodosius Dobzansky (1970) "nothing in biology makes sense except in the light of evolution"; but it is also true that very little in evolution makes much sense without a phylogenetic context (Doyle and Gaut 2000).

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		Mouse	Rat	Sheep	Chicken
Leptin	**Mouse**	100% (501)	95.6% (501)	83.3% (438)	94.6% (501) *
	Rat		100% (501)	83.6%	90.4% (501) *
	Sheep			100% (438)	81.5% (492) *
	Chicken				100% (492) *
Prolactin	**Mouse**	100% (678)	89.4% (678)	68.4% (687)	62.6% (689)
	Rat		100% (678)	71.3% (689)	65.7% (647)
	Sheep			100% (687)	67.3% (688)
	Chicken				100% (687)
Interferon γ	**Mouse**	100% (465)	88.2% (471)	64.2% (466)	59.8% (391)
	Rat		100% (471)	65.4% (501)	56.4% (385)
	Sheep			100% (501)	57.2% (381)
	Chicken				100% (494)
Interleukin2	**Mouse**	100% (507)	85.6% (437)	65.6% (360)	No match
	Rat		100% (464)	67.5% (467)	No match
	Sheep			100% (468)	55.4% (186)
	Chicken				100% (430)

* Chicken leptin sequence as per Taouis *et al.* (1998)

Table 6.1. A comparison of percentage similarities, at the nucleotide level, between cytokine genes of the mouse, rat, sheep, and chicken. Percentage similarities were obtained using LOCALFASTA (Wisconsin Package, Version 8, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711) with a word size of 2 or 6 with nucleotide sequence corresponding to the published coding region of the mouse, rat, sheep and chicken cytokines leptin, prolactin, interferon gamma and intefleukin 2 (accession numbers; U18812, U48849, U84247, AFO12727, X02892, V01249, M27057, 304614, K00083, AF010466, X52640, U27465, M22899, X01772, *X55641*, AF033563). Results are presented as the percentage similarity of the number of nucleotides for the different comparisons. Apparent discrepancies in the number of bases (given in brackets) are due to the inclusion of gaps in the comparison.

Phylogenetic inference depends on similarities between species both in morphological characters and in molecular sequences. At the molecular level, information can be obtained by comparing sequences of amino acids and their molecular configurations in different proteins, as well as by comparing sequences of nucleotides in various DNA and RNA molecules.

Inherent in all the various phylogenies is the concept that evolutionary differences between organisms arise from mutational differences, and therefore, in general, the greater the number of mutational differences between organisms, the greater their evolutionary distance. Evolutionists have used this concept to furnish the evolutionary time scales; that is, they have assumed that mutations are incorporated (or fixed) at fairly regular rates over time, and the degree of mutational distance for a phylogenetic interval therefore correlates with the time in which such phylogenetic evolution took place. In other words, regarding changes in a specific gene, this assumption suggests that an evolutionary clock at the molecular level determines the rate at which mutations become fixed. Since fixation of these mutations mostly depends on the clock rather than on their adaptive or selective value, Kimura (1968) and King and Jukes (1969) have independently proposed that mutations are primarily "neutral" in their effect. The "neutral mutation-random drift" theory implies that some features of macromolecules are relatively unimportant, so that a significant number of mutations are possible and can be fixed in the population by random drift. Sometimes they consider all or nearly-all possible silent mutations as neutral which would imply that no appreciable selection is operating on the mRNA itself. Whenever selection operates, deleterious mutations create a "genetic load" for the organism. The theory is radical because it stipulates that most evolutionary change is invisible to natural selection and therefore evolutionarily neutral.

Under the stochastic process of genetic drift, a new neutral mutation — which is typically found in only one individual in a population — will usually be lost to evolution, but occasionally by chance a neutral mutation can become the predominant variant in a population. In fact, the neutral theory of molecular evolution neither ignores nor constitutes an alternative to natural selection. Instead, it assumes that negative selection

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or selective constraint is a potent force in the evolution of genes (Kimura 1983). Kimura also recognized that positive (or adaptive) mutations must occur and can motivate evolutionary change. The radical aspect of neutral theory is the assertion that positive selection events are rare relative to the number of evolutionary changes brought about by neutral mutations and random genetic drift (Kimura and Ohta 1974). On the whole, this assertion has proven to be true at the molecular level (Doyle and Gaut 2000). Thus, a phylogeny derived from the mutational distance data provides an average number of nucleotide substitutions at each branching point that corresponds with a linear relationship to time of divergence.

The data so far show two different rates at which such substitutions have incorporated: a slow rate of divergence for humans, apes, and birds, and a faster rate for rodents, lower primates, Drosophila, and sea urchins (Britten 1986). Britten suggests that the slower rates of divergence in the lower mutation rate would result from improved DNA repair systems. Such lower mutation rates would be advantageous in groups that evolved toward increased parental investment in their offspring- that is, reduced birth rate and greater postnatal care. However, Wilson and co-workers (1987), in contrast, argue that many different genes in both bacteria and mammals shows fairly similar rates of nucleotide substitutions at synonymous codon sites. In either case, it does not affect the present hypothesis of divergence between birds and mammals.

In earlier studies immunological and hybridization techniques, and restriction fragment length polymorphisms (RFLP) were used to estimate genetic variations in populations (Nei 1994). More recently, evolutionary analysis of DNA sequence data has involved analyses of two or more sequences that are hypothesized to be homologous. These sequences are aligned by determining which positions along the DNA or protein sequences are derived from a common ancestral position — in other words, which positions are homologous within a set of homologous genes. The methods employed in alignment are the pairwise algorithm of Needleman and Wunsch (1970) that form the core of most multiple sequence alignment programs, Markov Chain Monte Carlo

methods (Eddy 1996), and methods that simultaneously consider alignment and phylogeny (Hein 1989; Wheeler and Gladstein 1994).

In the distance method, as used in the present analysis, an average distance measure for each pair of species is considered. Genetic distance, defined as the extent of gene differences between populations or species that is measured by some numerical quantity (Nei 1987), is assumed to be a measure of the evolutionary divergence between copies of homologous genes that share a common ancestor. Thus, it is dependent on the mode and frequency of the mutations that have occurred from the time of the common ancestor (Beaumont et al 1998). The distance approach to phylogeny reconstruction begins with estimation of pairwise distances between nucleotide sequences. Pairwise distances compensate for multiple hits (occurrence of two or more substitutions at the same site) by transforming observed percent differences between aligned sequences into an estimate of the actual number of nucleotide substitutions, using one of the various models of molecular evolution.

In practice, the stochastic nature of DNA sequence substitutions is modeled using the tools of probability and statistics. Many variations of the model have been formulated, including models that permit different probabilities of change among bases (Kimura 1980; Tamura and Nei 1993), codon-based model of nucleotide substitutions (Goldman and Yang 1994), models that assume that some nucleotide sites evolve more rapidly than others (Yang 1996), and models that distinctly partition amino acid altering (nonsynonymous or 'replacement') nucleotide substitutions from non-amino-acid altering (synonymous or 'silent') nucleotide substitutions (Nei and Gojobori 1986; Goldman and Yang 1994; Muse and Gaut 1994). Nucleotide substitution models form the basis of statistical inference in distance-based and maximum-likelihood phylogenetic methods (e.g. Huelsenbeck and Crandall 1997; Huelsenbeck and Rannala 1997).

Substitution is the presence of different nucleotides in equivalent positions of homologous DNA or RNA sequences. Mutation is the initial change in an individual organism, while substitution is a change in a population. Substitution of nucleotide bases

in a codon without altering the amino acid it encodes is known as synonymous substitution. Substitution models have certain added advantages over simple counts of differences between sequences. First, the amount of information derived from substitution data is greater than that from simple comparison studies. Secondly, they allow the estimation of the actual — not just the observed — number of nucleotide substitutions that have occurred between sequences. Thirdly, nucleotide substitution models form the basis of statistical tests, which in turn, can be used to address phylogenetic questions, far beyond the question of genes evolving at different rates (Doyle and Gaut 2000).

Recently, with the enormous amount of sequence data available from various classes of species, mostly from the protein-coding regions of the genome, efforts to compare the substitution rates among genes or different DNA regions have been intensive. These studies have significantly advanced the knowledge of mechanisms responsible for the different rates of substitution during evolution (Ohta 1995). Obtaining a reliable estimate of the rate of nucleotide substitution requires that the degree of sequence divergence must be neither too small nor too large. Additionally, the allelic divergence (polymorphisms) that occurred within the ancestral population prior to the divergence event also must be taken into consideration (Takahata and Satta 1997).

The three major attributes affecting the substitution rates are (1) mutational inputs, (2) functional constraints, and (3) positive selection (Graur and Li 2000).

In the vast majority of genes, the synonymous substitution rate greatly exceeds the nonsynonymous rate (almost 25 times). It is noted that the substitution rate in a gene is highest at fourfold degenerate sites; slightly lower in introns and the 3' flanking region; intermediate for the 3' untranslated region, the 5' flanking and untranslated regions and twofold degenerate sites; and lowest at nondegenerate sites (Graur and Li 2000). It is less clear why synonymous substitution also changes from gene to gene. However, chromosomal position of the gene (Wolfe et al. 1989), selection against some synonymous mutations, and the nucleotide

composition at an adjacent position (Ticher and Graur 1989) are some of the factors attributed to this phenomenon.

An interesting example of the effect of the mutation rate on the rate of substitution is the so-called 'male driven evolution'. The number of germ cell divisions from one generation to the next in males is approximately 6 times higher than in females (Chang *et al.* 1994) due to the difference between oogenesis and spermatogenesis. Thus, for all pair-wise comparisons between an X-linked and a Y-linked zinc-finger protein-coding genes, Shimmin *et al.* (1993) found that Y-sequences were more divergent, i.e., have evolved faster than their X-homologues. Generally, in rat, mouse, hamster, and fox, the mutation rate in males was found to be twice as large as in females (Lanfear and Holland 1991; Chang et al. 1994). However, McVean and Hurst (1997) argued that the higher rate of nucleotide substitution in Y-linked sequences is due to a reduction in the mutation rate in the X-chromosome in comparison to that in the Y-chromosome and autosomes, because of a lesser exposure of the X-chromosome to deleterious recessive mutations in the heterozygous condition. To test this possibility as an alternative to the male-driven evolution, Ellegren and Fridolfsson (1997) studied mutation rates in birds, where males are homogametic (WW) and females are heterogametic (WZ). They found that male-to-female ratio in mutation rates ranged from 4 to 7, consistent with the male-driven evolution hypothesis. Thus it is valid to assume that male-driven evolution occurs in both mammals and birds (Graur and Li 2000).

Within the framework of the neutral theory of molecular evolution, the rate of mutation and the intensity of selection have to be considered to explain the large variation in the rates of nonsynonymous substitution among genes. Different regions of the genome may have different propensities to mutate. However, this alone cannot account for the large variation in the rate of nonsynonymous substitution. Thus, the most important factor in determining the rates of nonsynonymous substitution seems to be the intensity of purifying selection (selection against deleterious mutations), which in turn is determined by functional constraints. The intensity of purifying selection is determined by the degree

of intolerance to mutations, characteristic of a site or a genome region (Miyata *et al.* 1980). This functional or selective constraint defines the range of alternative nucleotides that is acceptable at a site without affecting the function or structure of the gene or the gene product negatively. DNA-regions such as protein-coding regions or regulatory sequences, in which a mutation is likely to affect function, have a more stringent functional constraint than regions devoid of function (Jukes and Kimura 1984). The stronger the functional constraints on a macromolecule are, the slower the rate of substitution will be. Indeed, pseudogenes, which are devoid of function, seem to have the highest rate of nucleotide substitution (Graur and Li 2000).

As far as protein-coding regions are concerned, there have been several attempts to quantify functional constraints independently of their rate of substitution. One such measure is the functional density (Zuckerkandl 1976). The functional density of a gene, F, is defined as ns/N, where ns is the number of sites committed to specific function and N is the total number of sites. F, therefore, is the proportion of amino acids that are subject to stringent functional constraints. The higher the functional density, the lower the rate of substitution. Thus, a protein in which the functional sites comprise a lower percentage of its sequence will be less constrained, and therefore will evolve more quickly than a protein with a larger percentage of its sequence, which is essential for its biological function. Therefore, functionally less important molecules or parts of molecules evolve faster than more important ones. This principle is frequently used in its opposite sense, where the rate of nucleotide substitution is used to infer the stringency of structural and functional constraints in a particular sequence (Graur and Li 2000).

Because the rate of mutation at synonymous and nonsynonymous sites within a gene should be the same, or at least very similar, the difference in substitution rates between synonymous and nonsynonymous sites may be attributed to differences in the intensity of purifying selection between the two types of sites. This is plausible in the light of the neutral theory of molecular evolution. The discrepancy between synonymous and nonsynonymous rates in protein-coding genes serves as a convincing demonstration of

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the inverse relationship between the intensity of the functional constraint and the rate of molecular evolution.

Comparative analysis of apolipoprotein sequences from various mammalian orders suggest that in these domains, exchanges among hydrophobic amino acids (e.g., valine for leucine) are acceptable at many sites (Luo *et al.* 1989). At the other extreme, since most of the amino acids in histone H3 interact directly with the DNA or other core histones in the formation of nucleosomes, there are very few possible substitutions that can occur without altering the function of the protein. As a consequence, H3 is highly intolerant of most amino acid changes. This protein is one of the slowest evolving proteins known, evolving more than 1,000 times more slowly than the apolipoproteins (Graur and Li 2000). Comparison of amino acids in haemoglobin and cytochrome-c also reveals the influence of functional constraint. Like haemoglobin, cytochrome-c also carries oxygen, binds haeme, and responds structurally to changes in physiological conditions; but in addition to these haemoglobin-like functions, this protein also interacts at its surface with two enzymes: cytochrome oxidase and cytochrome reductase. Consequently, the rate of amino acid substitution in this protein is lower than that of haemoglobin (Perutz 1983).

Within a protein, the different structural or functional domains are likely to be subject to different functional constraints and to evolve at different rates. Examples are insulin and haemoglobin. In case of insulin, the nonsynonymous substitution rates for the regions coding for the C peptide and the signal peptide, which do not take part in the hormonal activity of insulin, are about 5 and 6 times, respectively, higher than that in the A and B chains, which have important roles in the function of the hormone (Steiner and Chan 1988; Chan *et al.* 1992). In case of haemoglobin, the surface of the molecule performs no specific function and is constrained only by the requirement that it must be hydrophilic. On the other hand, the internal residues, especially the amino acids lining the haeme pocket, play an important function in the normal function of the molecule. Accordingly, purifying selection seems to operate in such a way that the residues on the surface of the molecule evolve 8-11 times faster than the residues in the interior. Mutations affecting

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the interior of the molecule have been shown to cause harmful abnormalities, whereas replacements of surface amino acids often do not result in any clinical effects (Perutz 1983).

Another example is the fibrinogens, a group of elongated proteins acting as the precursor of fibrin, an essential polymer in the clotting process. The highly soluble fibrinogens are converted into insoluble fibrins by the proteolytic action of thrombin, which cleaves apart short polypeptides called fibrinopeptides. Fibrinopeptides have little known biological functions on their own, and consequently, any change in amino acid that will still allow them to be cleaved will be acceptable (Doolittle *et al.* 1971; Doolittle 1990; Murakawa *et al.* 1993). Indeed, fibrinopeptides are among the fastest evolving proteins, whereas the biologically active fibrins evolve at relatively low rates (Graur and Li 2000).

The role of functional constraints can further be demonstrated in the acceleration of nucleotide substitution rates following partial loss of function of a gene. Such genes are subject to relaxation of selection where selection constraints are partially, rather than entirely, removed, as opposed to the pseudogenes, which have completely lost their function. Hendriks *et al.* (1987) sequenced the αA-crystallin gene in the blind mole rat and compared its rate of substitution with those in other rodents, which possess fully functional eyes. The blind mole rat αA-crystallin possesses normal functions and expression, but its nonsynonymous substitution rate is 20 times faster than the rate in the rat. Furthermore, the fact that the nonsynonymous substitution rates are still lower than that in pseudogenes, led the authors to suggest that αA-crystallin might not have lost all of its vision-related functions, which was not proved at that time. However, recently, it has been discovered that αA-crystallin also functions as a molecular chaperone that can bind denaturing proteins and prevent their aggregation (Bova *et al.* 1997; Mornon *et al.* 1998). Interestingly, the sites involved in the chaperone activity are conserved in the blind mole rat. This provides a classical example where the functional constraints have been relaxed considerably, which in turn hasten the nonsynonymous substitution rates.

Finally, Zhang *et al.* (1997d) and others have devised tests to detect departures from the neutral mode of molecular evolution. In some immunoglobulin genes these show that nonsynonymous rates in the complementarity-determining regions are higher than the synonymous rates. The higher rate has been attributed to overdominant selection for antibody diversity (Tanaka and Nei 1989). Nevertheless, for the entire immunoglobulin gene, the nonsynonymous substitution rate is considerably lower than the synonymous rate. This observation shows that, even in immunoglobulins, most nonsynonymous mutations are detrimental and are eliminated from the population. Hughes and Nei (1989) reported a similar case in major histocompatibility complex genes, i.e., the rate of nonsynonymous substitution exeeds the rate of synonymous substitution, attributed to overdominant selection. However, according to a survey by Endo *et al.* (1996), positive selection affecting entire protein-coding sequences is suspected in only very few cases (about 0.45% in a total of 3,595 groups of homologous sequences studied). Nevertheless, positive selection affecting parts of genes or individual sites within the gene might have been unnoticed in this survey, since only whole genes were considered.

As explained above, among the prominent features of the neutral theory of molecular evolution, the following two are particularly noteworthy in the context of this thesis: (1) for a given protein, the rate of evolution is roughly constant per year, and (2) the molecules or parts of molecules that are subject to less functional constraints evolve (in terms of mutant substitutions) faster (Kimura 1977; Jukes and Kimura 1984; Graur and Li 2000). Accordingly, it is possible that not all synonymous mutations are neutral, but the possibility is very high that, on average, synonymous changes are not subject to natural selection. It further suggests that a functional constraint diminishes the rate of evolution converging to that of the synonymous substitutions. Such a convergence (or plateauing) of molecular evolutionary rates is, in turn, considered to be strong supporting evidence for the neutral theory. Practically, estimation of synonymous and non-synonymous substitution rates is important in understanding the dynamics of molecular sequence evolution (Kimura 1983). Since the rate of synonymous substitution is much higher than that of non-synonymous substitution and is similar for many different genes, synonymous substitution may be used as a molecular clock for dating the evolutionary time of closely related

species (Kafatos et al 1977; Kimura 1977; Miyata et al 1980; Li et al 1985; Ina 1996) including chicken (Perler et al 1980).

Drawing upon these theoretical considerations, the objective of the work described in this chapter are:

(1) to compare phylogenetic trees based on the rate of synonymous substitutions in the prolactin and leptin sequences, and

(2) to compare statistically the synonymous substitution rate between chicken and mouse leptin with the results of twenty randomly chosen gene sequence pairs from chicken and mouse.

6.3 Methods

6.3.1 Construction of phylogenetic trees for prolactin and leptin

6.3.1.1 Sequences, programme and algorithm

Prolactin and leptin sequences from five species as detailed in table 6.2 were selected to construct the phylogenetic trees. The evolutionary trees for the prolactin and leptin gene were created using 'GrowTree' function of the Wisconsin GCG programme, version 10.0. GrowTree creates a phylogenetic tree from a distance matrix created by Distances using neighbor-joining method. It accepts a distance matrix in the format produced by the programme, 'Diverge'. 'Diverge' measures the number of synonymous and non-synonymous substitutions per site of two or more aligned protein coding regions and can output matrices of these values (see below). 'GrowTree' constructs a tree from a matrix of synonymous or nonsynonymous substitutions. 'Neighbor-Joining' method is designed to find an approximation to the minimum evolution tree for a set of aligned sequences, using less computer time than the full algorithm for determining a minimum evolution

No.	Organism	Accession	Selection	
			From	To
1	Gallus gallus	J04614	157	729
2	Homo sapiens	NM000948	116	688
3	Mus musculus	X02892	161	733
4	Monodelphis domestica	AF067726	166	738
5	Ovis aries	M27057	211	783

No.	Organism	Accession	Selection	
			From	To
1	Gallus gallus	AF012727	55	489
2	Homo sapiens	U18915	1	504
3	Mus musculus	NM 008493	64	501
4	Sminthopsis crassicaudata	AF159713	132	569
5	Ovis aries	U84247	1	438

Table 6.2: The coding sequences of the leptin and prolactin genes used to reconstruct the phylofenetic trees. Source species, Genbank accession number, and the region of selection of the coding region from the published sequence are given in the table. On the top, the five sequences are of prolactin and in the bottom, the five sequences are of leptin.

tree. It works best when the distances are additive. The algorithm is that of Saitou and Nei (1987) supplemented by Studier and Keppler (1988) and modified by Swofford et al. (1996).

The neighbour-joining method clusters the sequences in a pairwise fashion. However, instead of picking the next pair to cluster by looking for the smallest distance in the distance matrix, this method seeks to form pairs that minimize the sum of the branch lengths for the entire tree. Therefore at each round of clustering, all possible pairs of entries are considered one at a time and the sum of the branch lengths for the resulting tree is calculated. The pairing that results in the smallest sum is the one that will be used to form the new cluster. This new cluster replaces its two constituent entries in the distance matrix (reducing the dimension of the distance matrix by one), and distances are calculated between the new cluster and the remaining entries in the distance matrix. The process continues until only two entries remain. The resulting tree is an unrooted tree.

All the five prolactin sequences (coding region only) were entered into a single project directory in GCG format by the function 'Seqed' which enters the sequence bases in the GCG-compatible format that enables further analysis. Similarly, all the five leptin sequences (coding region only) were entered in another project directory with 'Seqed' function. The alignments were prepared with the 'localpileup' function (alignment of the corresponding sequences to enable the pairwise comparison) and verified by 'lineup' (lining up the corresponding sequences one upon the other showing the exact matches and gaps). The 'Diverge -tof' function (analysing the pairwise distance measurement and calculating the nonsynonymous [Ka] and synonymous [Ks] substitution rates, the ratio of nonsynonymous to synonymous [Ka/Ks] substitution rates) was used to create the distance matrix, with output separately into Ks and Ka files. Using the synonymous substitution as the input file, a phylogenetic tree was by the 'Growtree' function (as mentioned above).

6.3.2 Analysis of synonymous substitutions in mouse and chicken genes

6.3.2.1 Sequences, programme and algorithm

Random numbers were obtained using Microsoft Excel 5.0. Chicken sequences obtained from Genbank were numbered 1 to 1,073 in alpha-numeric descending order from A1438163 to Z94720. Twenty sequences were selected (table 6.3) from these using the random numbers and used for further analysis. Mouse sequences homologous with the coding region of the selected chicken sequences were identified by name or the NCBI Blast programme (http://www.ncbi.nlm.nih.gov/blast/).

The programme 'Diverge' was used to estimates the pairwise number of synonymous and nonsynonymous substitutions per site between two or more aligned nucleic acid sequences that code for proteins. It uses a variant of the method published by Li et al (1985). 'Diverge' makes a pairwise codon-by-codon comparison of aligned protein coding sequences to estimate the number of synonymous and nonsynonymous substitutions. The program is based on the method described by Li et al., as modified by Li (1993)) and by Pamilo and Bianchi (1993)). It uses a translation table to determine codon degeneracies and applies Kimura's two-parameter method (1980) to correct for multiple hits and to account for the difference in substitution rates for transitions and transversions.

The method of Li et al. was originally implemented to study mammalian sequences using the universal genetic code or the mammalian mitochondrial code. The authors collected statistics on substitutions in mammalian genes to derive relative likelihoods of codon changes, which could be used to weight the substitution pathways. This method has several improvements over earlier methods. While the earlier methods assume that nucleotide substitution occurs randomly, this method allows differences in the transitional and tranversional substitution rates. It also corrects more rigorously for multiple substitutions by considering nondegenerate sites, twofold degenerate sites, and fourfold degenerate sites separately.

SI No.	CHICKEN			MOUSE		Synonymous Substitution (Ks)
	Accession	Gene	Accession	Gene		
1	D45416	Neuropilin	D50086	Neuropilin		123.59
2	X94742	Cor2 Oifactory receptor	AJ133428	Or37e gene		162.49
3	AF131057	Substance P Receptor	X62934	Substance P Receptor		91.24
4	X89507	AMPA Receptor	AB022913	M-glutamate receptor channel os4 subunit		74.81
5	M74057	Growth Hormone Receptor	M33324	High Mol. Wt. Growth Hormone Receptor		106.17
6	U37273	Winged Helix Protein CWH-2	Y08222	MFH-1 gene		100.34
7	AF041799	Insulin Receptor-related tyrosine kinase	AF056187	IGF-1 Receptor		123.13
8	M26810	Nerve Growth Factor	V00836	Nerve Growth Factor		127.03
9	AF082666	Interleukin receptor 2	U53696	Class II cytokine receptor 4		150.67
10	X65458	Stathmin	X94915	mRNA for Pr22 Protein		92.68
11	X04810	Carbonic anhydrase II	K00811	Carbonic anhydrase isoenzyme II		94.89
12	L21719	C-eyk protooncogene	L11625	Receptor tyrosine kinase		151.61
13	AF036942	Photoreceptor guanylate cyclase I	L41933	(Retinol) guanylyl cyclase		118.75
14	AF085248	Calmodulin-dependent protein kinase	X14836	Murine mRNA for Calmodulin kinase II		93.51
15	U20216	Inward Rectifier K channel	AF021136	Inward Rectifying K channel		113.89
16	L12695	Engrailed Protein (En-1)	Y00201	En-1 gene surrounding homeobox		57.92
17	U62143	Homeobox containing TF Hoxb-1	X53063	Hox 29 gene for homeodomain protein		82.89
18	AF071026	Truncated testis-specific box1 BPRCR	X73372	Prolactin Receptor		141.12
19	L18784	TGF-beta type II receptor	D32072	Isoform of TGF-b type II Receptor		150.21
20	AB002410	17-beta hydroxy steroid dehydrogenase	X89627	17-beta hydroxy steroid dehydrogenase		139.51
21	AF012727	LEPTIN	U18812	LEPTIN		6.82

Table 6.3: Synonymous substitution values (Ks) of random chicken genes and their mouse homologues. The coding regions of 20 random sequences from the published chicken genes were selected at random using random number generator of Microsoft Excel 5.0. The mouse homologues were obtained by the BLAST search of the NCBI world wide web site. With the help of Wisconsin GCG programme the sequences were aligned and lined up for checking the correctness of the frame. The sequences were then compared for evolutionary divergence in the GCG programme. Given in the table are the Genbank accession numbers, descriptions of the genes and the synonymous substitution values (Ks) per 100 sites. Also given the same details for leptin.

The method of Li *et al.* (1985): (1) scans a pair of sequences codon by codon and classifies the nucleotide sites into nondegenerate, twofold degenerate, and fourfold degenerate sites; (2) compares the two sequences codon by codon to classify the observed nucleotide differences; (3) calculates the proportion of transitional differences and the proportion of transversional differences; (4) uses Kimura's two-parameter method to estimate the true number of transitional and transversional substitutions at each type of site. (This step corrects for multiple hits and for differing substitution rates for transitions and transversions) and (5) computes K(s) (synonymous substitutions per synonymous site) and K(a) (nonsynonymous substitutions per nonsynonymous site).

The function 'diverge' in GCG version 10.0 was used to analyse the divergence of the sequences and to give a weighted number of synonymous substitutions/100 amino acids. The 'diverge' function in GCG (version 10.0) uses pairwise comparison to arrive at the distance estimate between the sequences aligned. It calculates the synonymous and nonsynonymous substitutions between the aligned sequences and expresses the rate for every 100 sites, which is equivalent to 300 nucleotides or bases. A score of 200 is the maximum that can be acceptable. Theoretically, this can be explained in the following way.

In a triplet codon, if one base is substituted with another one without causing a replacement of amino acid, it gives a synonymous substitution rate (Ks) of 1 per 1 substitution site, which is equivalent to a score of 100 per 100 substitution sites in the 'diverge' function of the GCG programme. In this way, a score of Ks 200 means there are 200 bases substituted out of 300, which in turn means two out of every triplet codon has been substituted, without altering the encoded amino acid. If the reported score surpasses this value of 200, it means that some of the codons have undergone complete substitution of all the three bases, and despite that, remained silent or synonymous. With the known codon usage, there is no amino acid known to be encoded by two different codons differing in all the three base positions between them. In this way any score more than 200 from the 'diverge' analysis of DNA sequences should be rejected as erroneous. In the

present analysis all the synonymous substitution (Ks) scores obtained were well below this limit and hence were accepted.

6.3.2.2 Analysis

All the chicken and mouse sequences were saved in GCG format. Each of the pairs of these corresponding chicken and mouse sequence files was checked by 'translate' (translating the nucleotide sequence to amino acid sequence) to ensure that the protein encoding frame was correct. The sequences were then aligned using 'localpileup' (as mentioned above) and verified by 'lineup' (as mentioned above) functions. Using another command 'diverge' (analysing the pairwise distance measurement and calculating the nonsynonymous [Ka] and synonymous [Ks] substitution rates, the ratio of nonsynonymous to synonymous [Ka/Ks] substitution rates) the synonymous and nonsynonymous substitution rates were obtained. The synonymous substitution values per each 100 amino acids (300 nucleotides) were obtained from the respective 'diverge' files.

6.4 Results

6.4.1 Phylogenetic trees

When the evolutionary phylogeny of leptin was compared with that of another cytokine, prolactin, based on synonymous substitution of nucleotides during evolution, the position of chicken in the prolactin tree agrees with the consensus view of vertebrate evolution (Ridley 1993; Strickberger 1996). By contrast, the position of chicken using the published leptin sequence suggests divergence from the rodent lineage and is contrary to the accepted view of evolution (figure 6.1).

6.4.2 Synonymous substitution data analysis

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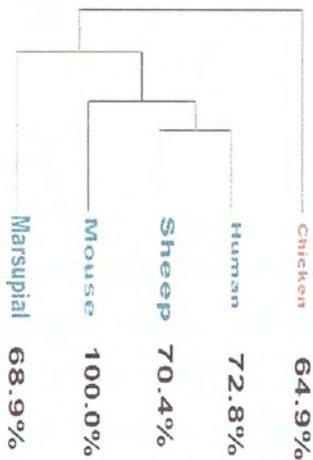
Figure 6.1 - Reconstructed phylogenetic tree for prolactin and leptin. The synonymous substitution values between the mouse and the chicken were used to construct phylogenetic trees originally by the 'Growtree' function of the GCG programme as mentioned in section *6.3.1.3*. Shown in the figure is the redrawn version of the original graphical output by the GCG analysis. The similarity of each sequence with respect to the mouse sequence is given in percentage.

The details of the random chicken and mouse genes are given in table 6.1. Log_{10} transformation of the data provides the best approximation to normality for the rate of synonymous substitution between 20 randomly selected pairs of chicken and mouse genes (figure 6.2). Leptin lies clearly outside this distribution. The probability of fitting (finding) the synonymous substitution value of leptin in this normal distribution is 1×10^6 (see appendix and figure 6.2).

6.5 Discussion

The phylogenetic tree reconstruction shows that the prolactin tree agrees with the presently accepted evolutionary divergence of birds and mammals dating back 300 million years (Ridley 1993; Strickberger 1996) whereas that of the published chicken leptin does not. Moreover, the degree of homology between the trees for mammalian leptin and mammalian prolactin, both including a marsupial, is in good agreement with the evolutionary histories of these species (Table 6.1). These observations suggest that any incongruity between gene trees and taxon (species) trees has not intruded in the statistical analysis of synonymous substitution values used in this thesis.

The analysis presented considers the synonymous substitution between chicken and mouse sequences randomly chosen from a list of 1,076 genes, thus representing an unbiased set of data. Almost identical results were obtained using genes, which were not picked at random, but were genes for which sequences were readily available for comparison. The nucleotide sequence data supports the view that phylogeny derived from the mutational distance provides an average number of nucleotide substitutions at each branching point that corresponds with a linear relationship to time of divergence (Fitch and Langley 1976). The present analysis suggests that the published leptin sequence has evolved more slowly than predicted in the chicken lineage.
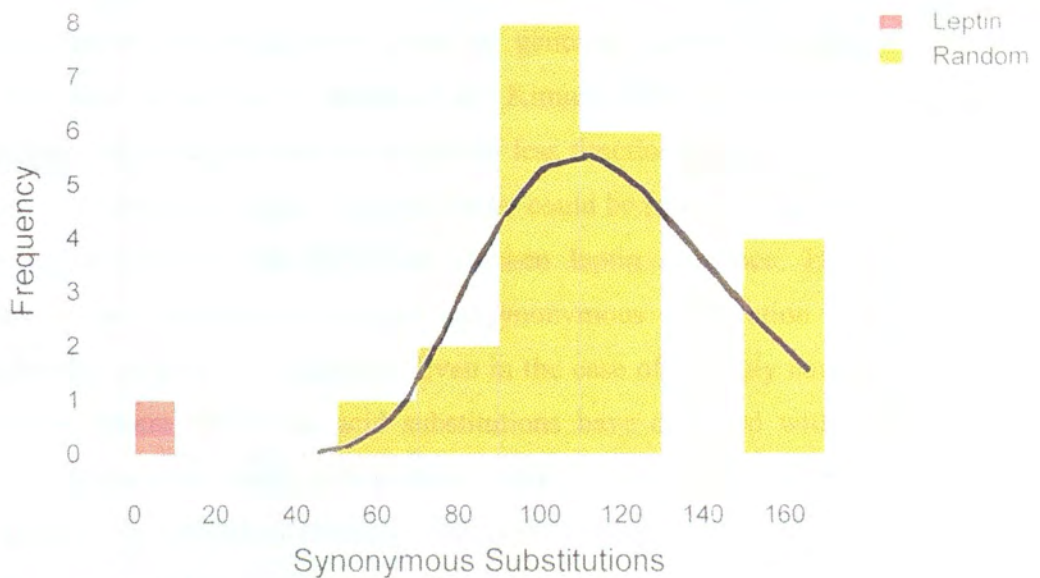
Figure 6.2 Frequency distribution of synonymous substitution values (Ks) of random sequences (n = 20) and leptin sequence between the mouse and the chicken. The yellow bars represent the frequencies of the random genes and the red bar (towards the left) is that of leptin. Also given the curve showing the normal distribution of the Log_{10} (Ks) superimposed. Accordingly, the chances of finding (fitting) the Ks value of leptin in this normal distribution is 10^{-6}, that is only 1 in 1million (see appendix for the calculation).

From another point of view, it is also observed that different proteins and stretches of DNA evolve at different rates (Ridley 1993). This may explain the differences between the rates of evolution of the leptin gene and other genes including prolactin. Mitochondrial DNA and ribosomal RNA are two extreme examples of the most rapidly and slowly evolving sequences, respectively. The difference is believed to be due to the variation in the available repair enzymes between mitochondrial and nuclear DNA, and the bulk of non-functional (neutral) rRNA, which are not protein coding. In addition to these extremes, there can be different genes or genomic regions that are prone to differential selection pressures as proposed by Kimura (1977) suggesting that the molecules or parts of molecules that are subject to less functional constraints evolve (in terms of mutant substitutions) faster. This possibility could be raised to argue the case for the extreme conservation of the published chicken leptin sequence. However, the extremely low values of synonymous and nonsynonymous substitution rates in the putative chicken leptin gene is exceptional. Even in the case of a highly conserved gene such as histone4, where the amino acid substitutions have occurred with the lowest known rate (Grunstein *et al.* 1976), synonymous mutant substitutions have occurred at a rate higher than in the published chicken leptin gene. These considerations allow the conclusion that the published chicken leptin sequence is of murine origin and is a PCR artifact. The only other formal possibility is horizontal transfer of DNA between mouse and chicken. This is not supported by the Southern analysis.

Thus, the present analysis demonstrates that the published chicken leptin sequences (Taouis et al 1998; Ashwell et al 1999) are improbable. The result of this analysis is consistent with the observation from chapter 4, that the leptin-like sequence in the chicken genome is less than 75% identical to that of the mouse leptin gene.

In retrospect, if the molecular evolutionary/phylogenetic analyses described above had been done first, it would have changed the strategies adopted in my experimental programme. Homology-cloning of leptin gene, which is only moderately conserved among the mammals, can now be seen to be an over-optimistic cloning strategy. Consideration of the functional constraints relevant in the differences in lipid metabolism between mammals and birds (e.g.,

adipocyte versus hepatocyte, Simon *et al.* 1991) suggests that the biology of chicken leptin might be significantly different from that in mammals and further reinforces the view that avian and mammalian leptins have low homology. The design of earlier experiments, especially the PCR amplification of a leptin-homologue from the chicken templates could have been improved. Degenerate primers, touch-down conditions and titration with varying concentrations of magnesium deployed described in chapter 3 were too strongly based on the mouse leptin sequence. More relaxed touch-down conditions and the wider range of degenerate primers should have been employed. Additionally, special attention could have been paid to the codon-usage preference in the chicken. Attention could also have been more focussed on regions of the leptin gene exhibiting lower nonsynonymous to synonymous substitution ratios, which may imply more functional constraints in operation, which would identify functionally conserved sequences. It could then have been possible to target these sequences for the design of PCR primers to amplify a leptin 'homologue' from chicken templates.

Information on synonymous substitution presented in this chapter could have been useful in choosing a non-homology strategies such as those suggested in section 7.2 (e.g., positional cloning) to clone a leptin-like candidate in the chicken.

CHAPTER 7

GENERAL DISCUSSION

7.1 Background, results and inferences

The aim of the thesis was to establish the existence of a leptin gene homologue in the genome of the domestic chicken (*Gallus gallus*) and to determine the degree of homology of the sequence with the mammalian leptin genes.

As detailed in chapter 1, positional cloning of the mouse leptin gene in 1994 (Zhang *et al.* 1994) opened up new avenues in the knowledge of energy homeostasis in general and in obesity research in particular. In that paper, the authors presented a Southern zooblot showing cross-hybridization between the coding region of the mouse leptin gene and DNA from several vertebrate species, including the chicken (figure 1.8). Sequencing of the human adipocyte cDNA clone which hybridized to the same probe showed that the nucleotide sequences encoding human and mouse leptin are highly homologous (Zhang *et al.* 1994). It was therefore suggested that the sequence of leptin gene is conserved among the vertebrates, although direct sequence information was not available to support this at that time.

After the leptin gene was cloned in the mouse and human, it was also cloned by RT-PCR in several mammals including the rat (Murakami and Shima 1995), rhesus monkey (Hotta *et al.* 1996), domestic pig (Bidwell *et al.* 1997), sheep (Dyer *et al.* 1997), the Israeli sand rat (*Psammomys obesus*) (Walder *et al.* 1997), and cow (Ji *et al.* 1998). The sequences from these species confirmed that the coding region of the leptin gene is conserved among these mammals (see figure 3.1).

A chicken leptin cDNA sequence was deposited in the Genbank database in August 1997 (Genbank accession: AF012727), which was about 70% homologous with the mouse

leptin cDNA. This sequence was updated in October 1997 changing about 30% of the bases. Surprisingly the revised chicken leptin sequence shares 95% identity at the nucleotide level with mouse leptin cDNA (Taouis *et al.* 1998). These authors cloned the putative chicken leptin gene from fat and liver of broiler chickens by RT-PCR using primers based on the sequence of the mouse leptin gene. Their paper did not demonstrate the existence of a leptin homologue in the chicken genome by showing Northern or Southern blots. The forward primer (Taouis-for, 29mer, section 2.17.3) used by these authors to clone the published chicken leptin sequence extends into the untranslated region of the mouse gene by 8 bases (see figure 3.2, where the remaining 21 bases have been shown). Inexplicably, the first 7 of these 8 bases do not match the corresponding mouse sequence (accession numbers U36238, U52147).

In 1999, another team of workers published a chicken leptin cDNA sequence identical to the earlier one reported by Taouis *et al.* (1998) with only a single base change (C for T) at position 342 (Ashwell *et al.* 1999a). Here also the cloning was done by RT-PCR, from liver and adipose tissue of broiler chicken. Southern and Northern hybridization blots were presented as evidence for the existence, and expression, of the gene in the chicken. However, the exact sizes of the sequences hybridizing to the leptin probe, the sequences of the probes used, and the hybridization conditions in the Northern and Southern analyses were not reported. Ashwell *et al.* (1999b) also demonstrated a direct effect of GH on leptin gene expression in chicken liver, but not in fat. The latter conclusion was reached using RT-PCR. This observation was based on a group size of 4-5 with a level of significance of $P < 0.05$. It is surprising that the authors did not sequence the fragments amplified and chose to use RT-PCR to measure leptin gene expression since they presented evidence of strong northern hybridization with the leptin probe (Ashwell *et al.* 1999a). A northern analysis would have been more conclusive. This suggests that the level of leptin gene expression in the chicken liver is too low to be reliably detected using northern hybridization.

In view of the high percentage of sequence identity with the mouse leptin, it is surprising that neither Taouis *et al.* (1998) nor Ashwell *et al.* (199a,b) showed that the 3' or 5'

flanking sequence of the putative leptin gene they claim to have cloned are from the chicken. In two years since the first publication of the putative chicken leptin cDNA sequence, there has been no published information (in Journals or gene databases), - on the non-coding genomic sequence of the chicken leptin gene. In view of these observations, the work for this thesis, which was begun before publication of the papers of Taouis *et al.* (1998) and Ashwell *et al.* (1999a,) was continued, but with the additional objective of confirming these authors' findings.

As mentioned in chapter 3, the entire the mammalian leptin coding region was taken into consideration when designing the primers to amplify a homologous sequence from chicken templates. Although the rodent (mouse and rat) leptin sequences formed the primary basis for the primers used in these experiments, degenerate primers based on a consensus of all known mammalian leptin sequences were also used in attempts to amplify chicken leptin gene by RT-PCR (figure 3.1 and table 3.1). Different batches of reagents and a wide range of fidelity and stringency conditions were also investigated in attempts to obtain a PCR product of the size predicted for leptin. In general, the results were disappointing. Promising amplified PCR products were sequenced (see section 3.3), but all were found to be unrelated to leptin or to any genes in the databases.

The same PCR primers and conditions as described by Taouis *et al.* (1998) using RT-RNA provided by the authors produced a PCR product of the predicted size for leptin but the sequence was unrelated to any sequence in the databases (unpublished observation, T. Boswell). Attempts to confirm this observation using RT-RNA prepared from liver and adipose tissue at Roslin failed to generate a PCR product using the conditions described by Taouis *et al.* (1998) (unpublished observation, T. Boswell) PCR was also carried out under a wide range of conditions with the primers specified by Ashwell *et al.* (1999a) using broiler and bantam chicken fat and liver cDNAs. These did not yield any PCR product of the predicted size (T. Boswell, unpublished observation).

Before work on this thesis began, mouse and human leptin genes were "cloned" from chicken templates by PCR amplification in this laboratory (Boswell, T., Dunn, I.C.,

Roslin Institute, personal communications). It was assumed that these leptin sequences were the result of contamination in the PCR reactions. Sheep and pig leptin have also been "cloned" from avian mRNAs in some other laboratories (M. Friedman-Einat, The Volcani Centre, Israel; G. Graham, Animal Science Group, University of Western Australia, personal communications) and were also considered as being due to contamination in the PCR reaction. In this thesis, PCR amplification using primers based on mammalian leptin sequences, invariably, generated products of sequences unrelated to leptin (e.g. the ~378bp band and the ~170bp band in RT-PCR, see section *3.3.2*). In this context, the reported mapping of the chicken leptin gene by A Vignal's group is of particular interest (Pitel *et al.* 1999). Using sets of primers based on the putative chicken leptin sequence reported by Taouis *et al.* (1998) a PCR product was obtained of the appropriate size for leptin. The gene represented by the PCR band, was mapped to a microchromosome, at a position that was not predicted from a syntenic comparison of the putative location of the leptin gene in mammals and birds. Following the publication of some of the work described in this thesis (Friedman-Einat *et al.* 1999) Vignal's group sequenced the PCR product that they used for their mapping study and found it was not a leptin sequence. They had not done this sequencing in their original paper (Pitel *et al.* 1999). Subsequently Vignal's group withdrew their observation on the mapping of the chicken leptin gene (Pitel *et al.*2000, in press).

These observations highlight the shortcomings of heterologous RT-PCR cloning. Genomic evidence is essential to ensure that a sequence obtained is derived from the target species. The failure to amplify a mammalian leptin homologue by PCR from chicken templates is consistent with a low homology between chicken and mammalian leptin cDNAs. It is possible that a complex secondary structure of the chicken leptin gene prevents PCR amplification. Even if this is the case, the homology between chicken and mammalian leptin cDNA must be lower than that reported by Taouis *et al.* (1998) and Ashwell *et al.* (1999a).

In view of the failure to clone chicken leptin by RT-PCR, evidence was sought for the existence of a mammalian-like leptin sequence in the chicken genome by Southern

analyses (chapters 4). Since the mammalian leptin coding region is only about 500 base pairs, random priming using ^{32}P as the reporter molecule (Ausubel *et al.* 1998) was used to ensure the production of a probe for Southern analyses with high sensitivity. In the Southern analyses two positive controls were included. The first was mouse genomic DNA which contained a leptin sequence with 100% identity to the mouse leptin probe, and the second was sheep genomic DNA containing a leptin sequence with 83% identity. This made it possible to compare hybrid stabilities between the sequences tested, which in turn, provided valuable clues to their homologies.

Results from the Southern analyses of chicken gDNA showed multiple hybridization signals at low and moderate stringencies. At low stringencies, the signal pattern showed multiple bands many of which disappeared at moderate stringency. This suggested the possibility either of multiple copies of the leptin gene or of nonspecific hybridization. This observation is inconsistent with that of Ashwell *et al.* (1999a), who demonstrated a single band on their Southern blot. However, one of the Southern analyses presented in chapter 4 also shows a single band (figure 4.3b). However, it was impossible to compare Ashwell *et al.*'s observation with that in chapter 4 because they did not report the size of their band (Ashwell *et al.* 1999a).

Comparative Southern analysis demonstrated that the hybrid stability of the mouse leptin probe with genomic DNAs from different species is in a descending order, mouse > sheep > chicken (chapter 4). This shows that the chicken leptin cDNA is not as homologous with the mouse leptin cDNA as is reported by others (Taouis *et al.* 1998; Ashwell *et al.* 1999a). Mathematical calculations based on known homologies between chicken and mammalian genes (see appendix) support this conclusion, predicting that chicken leptin cDNA is unlikely to be highly homologous with mouse leptin cDNA.

The Southern analyses described in chapter 4, indicated that there was a possibility of hybridization of a mammalian probe to the chicken genome albeit weak. This suggested that the mammalian probe would be potentially useful for screening recombinant chicken DNA libraries. The next phase of this thesis therefore explored the use of Southern

blotting to identify the chicken leptin gene in a chicken adipose tissue cDNA library (Stratagene). Surprisingly, this was not successful. The absence of hybridization signal may reflect a low level of leptin gene expression since the library was obtained from a bird deprived of food for 24h (Stratagene, personal communication). Next, a chicken genomic library in lambda phage vector was screened, and although this revealed some hybridization bands, none of them was specific for leptin. Finally a chicken genomic library in a cosmid vector (section 5.3) was screened and this produced strong hybridization signals, all of which were subcloned and sequenced (see tables 5.1 and 5.2 and figure 5.3a-j). None of these sequences showed homology with mammalian leptin sequences over more than a few base pairs, although these short stretches of identical bases in many of the sequences could have resulted in hybridization as mentioned in chapter 5.

Subsequently, in the Ph.D. supervisor's laboratory at Roslin, probes based on conserved human and pig leptin sequences in the 3' untranslated region were also used to screen the chicken genomic libraries in the phage and cosmid vectors. These too failed to identify sequences with a significant homology to leptin (T. Boswell, personal communication). These results further support the conclusion drawn from the PCR and Southern analyses, that there is low homology between chicken and mammalian leptin cDNAs.

This conclusion is supported by a theoretical analysis of the molecular evolution of leptin (chapter 6). Based on current knowledge of the rate of synonymous nucleotide substitution with evolutionary time, the probability that chicken and mouse leptin nucleotide sequences to be 95% identical, as reported by Taouis et al. (1998) and Ashwell et al. (1999a), is less than one in a million.

Studies on birds using mammalian leptin antibodies and leptin further support the view that there is low homology between avian and mammalian leptins. Immunocytochemical localization of leptin in the Roslin laboratory, using paraffin sections and an antibody to the N-terminal region of the human leptin, revealed reaction product on the periphery of fat cells in the mouse but not chicken (figure 7.1). No immunocytochemical labeling was
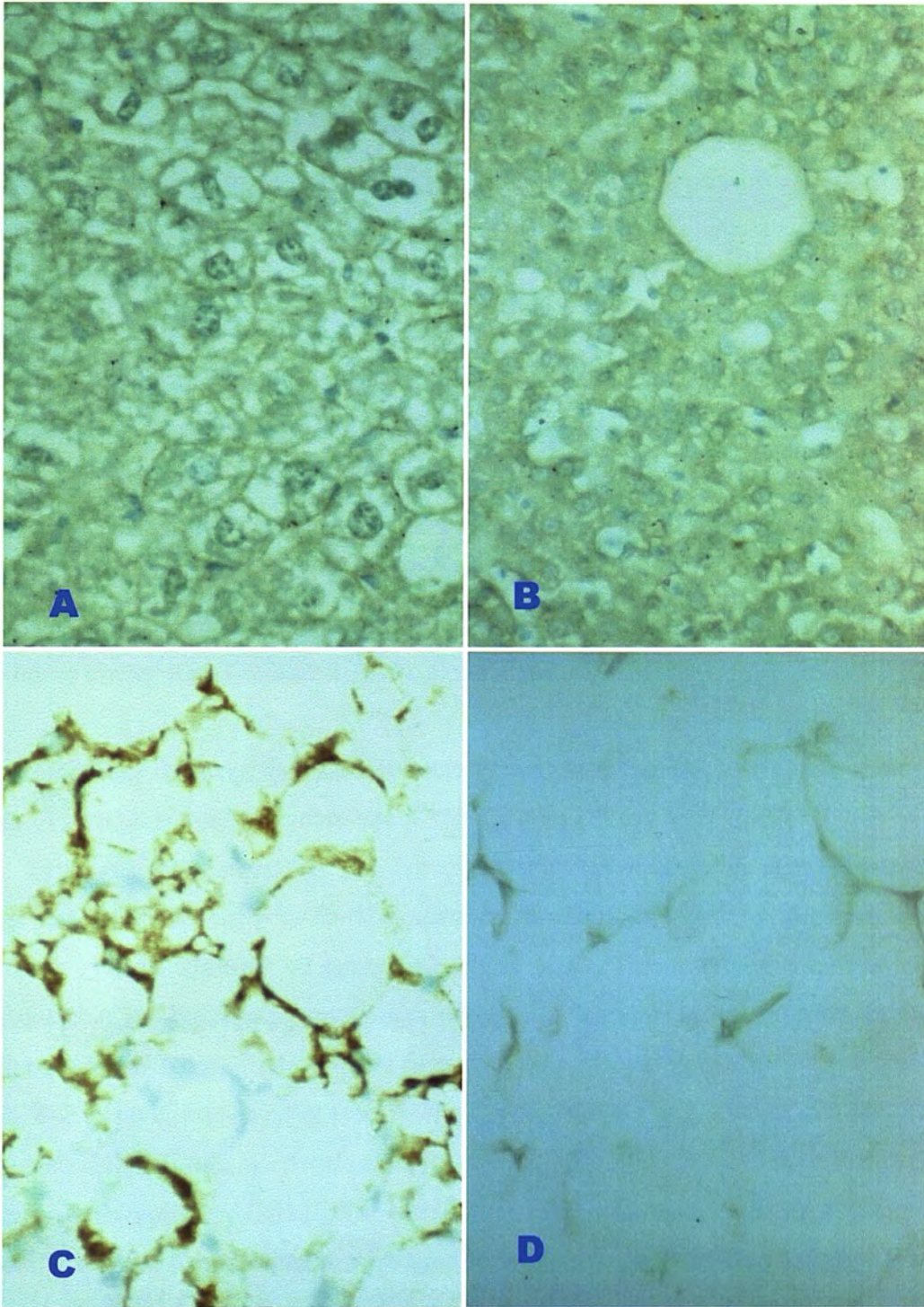
Figure 7.1: Immunocytochemical labeling of (A,C) mouse and (B,D) chicken (A,B) liver and (C,D) fat using paraffin sections and an antibody to the N-terminal region of human leptin. These observations were confirmed using an antibody to the C-terminal region of human leptin.

seen in mouse or chicken liver (figure 7.1). Observations on the physiological responses to the central administration of mouse leptin in birds have yielded conflicting results. For example, intracerebroventricular (icv) injection of mouse leptin does not influence food intake in male broiler chicken (Bungo *et al.* 1999). In contrast to this observation, systemic administration of recombinant (mouse-like) chicken leptin in broilers reduces food intake (Raver *et al.* 1998).

As detailed in chapter 1 (section 1.13), chickens regulate their body fat content: force-feeding cockerels induces obesity, but when the birds are allowed to feed freely, they reduce their food intake or stop eating until their body fat content returns to that in non-force-fed controls (Lepkovsky 1973). Consistent with this finding, food intake in cockerels is reduced after icv injection of concentrated plasma from fed, but not from fasted birds (Skews *et al.* 1984). These observations support the view that the control of adiposity in the chicken, as in mammals, involves an inhibitory action on the brain, by a blood-borne satiety signal, which could be avian leptin.

More compelling evidence for the existence of leptin in the chicken comes from the cloning of a chicken leptin receptor-like (COB-R) gene (Horev *et al.* 2000; Okhubo *et al.* 2000). This gene has a relatively low homology with its mammalian homologues: the average sequence similarity is 59-62% identical nucleotides; 49-51% identical amino acids; and 75-78% identical or conserved amino acids. This level of conservation is consistent with the estimated evolutionary divergence between birds and mammals and with the degree of evolutionary conservation of other cytokine receptors in these species. The COB-R gene is expressed in the chicken hypothalamus, lung, kidney, fat, and liver, as in mammals. However, the level of expression appears to be lower in the chicken than in mammals (Horev *et al.* 2000). The similarity in the pattern of tissue expression and conservation of the ligand-binding and signal-transducing motifs in the COB-R indicates conservation of the function of the mammalian leptin receptor and its avian homologue. When expressed in CHO cells, the protein product of COB-R binds to mammalian leptin, confirming that it encodes an avian leptin receptor (M. Friedman-Einat, personal communication). The COB-R gene has been mapped to a common syntenic group on

chicken chromosome 8 and human chromosome 1 (Dunn *et al.* 2000). This observation supports the hypothesis of a common evolutionary origin for COB-R and its mammalian homologue.

There are several lines of evidence that receptors for peptide hormones are evolutionarily more conserved than their ligands (Bolander 1989). For example, immunological experiments show that antibodies to the insulin receptor cross-react to those from many species whose insulins are immunologically distinct (Muggeo *et al.* 1979). Similar results have been obtained for the GH and prolactin receptors (Drake and Friesen 1981). Nucleotide sequence information from hormones and their receptors are consitent with the immunological data. For example, insulin and insulin-like growth factor-1 have a homology of 46% among vertebrates, but their receptors show a higher homology of up to84% depending on the domains compared (Stroud and Finer-Moore 1985). In a cross-species comparison between leptin and its receptor, it is possible that that the nucleotide sequence of chicken leptin should be less than 60% homologous with that of mammalian leptins (as the COB-R has 59-62% similarity with the mammalian counterparts, at the nucleotide level). This homology is lower than predicted from the Southern analyses of (chapter 4), but is consistent with difficulties in cloning the gene (chapters 3, 5).

The series of experiments described in this thesis could be seen as consistent with the view that a 'mammalian-like' leptin homologue is not present in the chicken. The failure to amplify a leptin-like sequence from the chicken templates, while amplifying the mouse sequences as positive controls, may be interpreted in this way. Similarly, Southern analyses, demonstrated that a leptin-homologue, if present in the chicken, it is less than 70% identical with the mammalian leptin sequences. This conclusion was supported by the failure of identifying leptin-homologues from the screening of chicken recombinant DNA libraries in various vectors. Analysis of the sequences from the PCR amplifications and cosmid library screening at the nucleotide and amino acid levels, paying particular attention to the functionally conserved sequences, also failed to identify any mammalian-like homologues with mammalian leptin-like genes. The absence of any functionally significant sequences from the nucleotide and amino acid analyses from chapter 3 and 5

(sections 3.3 and 5.3) is further consistent with the view that there are no leptin-homologues in the chicken genome. The moderate level of conservation of the leptin gene among the upper evolutionary cluster of mammals (chapter 6) suggests a low functional constraint operating upon the leptin gene, which may imply a lower conservation of this gene during the course of evolution. If this is the case, the chances of identifying any ancestor of the mammalian leptin gene sequence in the chicken are low. Physiological evidence for the existence of a leptin-like system in the birds is equivocal. Intracerebroventricular administration of mouse leptin in the chicken have not been found to induce consistent effects on feed intake (Bungo *et al.* 1999; Raver *et al.* 1998). The low molecular weight (< 1500) serum fraction from Leghorn cockerels fed ad libitum with a satiating effect in domestic chicken (Skewes *et al.* 1986) is incompatible with a larger leptin-like protein molecule.

The evidence for and against the existence of a chicken leptin gene with a functional homology to that in mammals is thus finely balanced. In order to resolve this issue, new strategies will be required in attempts to investigate the existence of a chicken leptin-like gene in future studies.

7.2 Future studies

7.2.1 Positional cloning of the chicken leptin gene

The leptin gene is mapped relative to a series of molecular markers on chromosome 6 in the mouse (Friedman et al. 1991; Walther et al. 1991; Bahary et al. 1993) and to chromosome 7 in man (Isse *et al.* 1995). Assuming that the chicken leptin gene occurs in the same syntenic group in chickens as it does in man and mouse, the predicted location for a chicken homologue of the mammalian leptin gene is on chromosome 1 between the *smoothened* homologue (SMOH) and the cell adhesion molecule (NrCM) genes (Groenen *et al.* 2000). This represents approximately a 200Mb region. If more genes which are predicted to lie closer to leptin than SMOH an NrCM can be mapped in the chicken, it may be possible to predict the putative location of the leptin gene with sufficient

precision to consider positional cloning using bacterial artificial chromosomes. Genes which may be adjacent to chicken leptin are BCP (violet cone protein, Genbank accession: M92039), cMET (Genbank accession: X84044), LAMB1 (Genbank accession: L00962), ARF5 (Genbank accession: X55998) and CAPZA2 (M80589). None has not yet been mapped in the chicken. The RFLP marker D6Rck 13 and another molecular marker Pax 4 (Genbank accession: AF118441), used in the positional cloning of the mouse leptin gene (Zhang *et al.* 1994), might also be useful for the positional cloning of chicken leptin.

7.2.2 Interaction cloning

The interaction method is used for cloning cDNAs encoding proteins that interact with a protein whose coding sequences are known (Finley and Brent 1996), the success of which largely depends on the ability of the proteins involved to interact with each other. Knowledge of the sequence information of the putative chicken leptin receptor may thus be exploited to clone chicken leptin gene by this method. In the case of chicken leptin, an adipose tissue cDNA library from *ad libitum* fed birds, constructed in an appropriate expression vector, could be used to translate the leptin DNA sequence in all three possible reading frames. Nucleic acid hybridization could then be combined with expression screening using labeled recombinant derived chicken leptin receptor protein, to reduce the number of false positives. The recombinant chicken leptin receptor protein could be synthesized by cloning the COB-R construct in an expression vector with necessary tags for purification and labeling. Interaction cloning has been successfully used in cloning of human cadherin-14 (Shibata *et al.* 1997), human prenylated rab acceptor (Bucci *et al.* 1999) and human Ro ribonucleoproteins (Bouffard *et al.* 2000).

If immobilization of the expressed cDNA library proteins on membranes inhibits its further interactions, the yeast two-hybrid system offers an alternative approach to cloning chicken leptin (Golemis *et al.* 1997). In vivo transcription-based assays for protein-protein interactions, such as yeast two-hybrid technique, are powerful methods to identify novel proteins based on their physical association with known proteins. In contrast to

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other methods, protein purification steps or development of antibodies are not involved. Moreover, the required cloning manipulations are minimal and the interacting partner is obtained as a partial or complete cDNA (Golemis *et al.* 1997).

In principle, the interaction trap is an extension of the two-hybrid system developed by Gyuris *et al.* (1993). This method employs the transcription of reporter genes as a synthetic phenotype to detect protein-protein interaction (Finley and Brent 1996). The two-hybrid approach chiefly exploits the modular domain structure of eukaryotic transcription factors. Many eukaryotic transcription activators have distinct functional domains for binding to specific DNA sequences and for activating transcription (Keegan *et al.* 1986; Hope and Struhl 1986). These domains can be exchanged from one transcription factor to another and still retain function, and thus, the resulting hybrid (or fusion) protein can activate transcription of genes, even if not bound covalently (Ma and Ptashne 1988). Practically, yeast transcription can be used to detect the interaction between two proteins if one of them is fused to a DNA binding domain and the other one is fused to an activation domain (Fields and Song 1989). When these interacting proteins come in close proximity, the DNA binding and transcription-activating domains come in close proximity too, which in turn can activate a reporter gene The vector for expression of a protein of interest fused to the appropriate domain of a transcription factor is called as the 'bait'. First, the known protein is expressed fused to the DNA binding domain of a transcription factor (bait fusion protein). Then a cDNA library to which the sequence for activation domain of the same promoter is fused (called an activation-tagged cDNA library) is expressed. Interaction of the bait fusion protein with the activation-tagged cDNA library results in transcription of a reporter gene (mostly nutritional genes which enable the survival of the yeast in specific media). In other words, only clones, which contain interacting proteins, can survive since the hybrid activates a yeast gene necessary for survival on the medium on which it is grown.

Thus, the COB-R gene product could be used as bait for ligands from chicken adipose tissue cDNAs in a yeast expression library (Allen *et al.* 1995; Young 1998). Positive clones would be identified on the basis of expression of appropriate markers and

reporters. This method has been successfully used for cloning growth hormone and prolactin receptors (Ozenberger and Young 1995). Expression of the bait could be confirmed by Western blotting using antibodies generated to an appropriate epitope of the chicken leptin receptor. This method has been successfully used for cloning growth hormone and prolactin receptors (Ozenberger and Young 1995).

7.2.3 Tissue distribution and physiological expression studies

Once the leptin gene is cloned, it would first be used to identify tissues in which it is expressed, with adipose tissue being targeted as the tissue of principal interest. Since more than 90% of total lipogenesis occurs in the liver in the chicken (Simon *et al.* 1991) this organ is also a potential source of leptin gene expression. Traditional RT-PCR and quantitative RT-PCR could be employed for this. Quantitative Northern analysis would be helpful in confirming the leptin gene expression producing added information on the transcript size and possible alternate splicing.

Physiological studies could be undertaken to establish whether expression of the leptin gene is regulated as in mammals. Initial studies would compare leptin gene expression in adipose and other tissues in fully fed and fasted birds, in relation to sex and developmental stage. Comparison between broiler and layers would be of particular interest because of differences in appetite and fat deposition. If differences are found, research on the effects of metabolic hormones such as insulin, glucagon, growth hormone, and corticosteroids would be informative.

In general, since leptin affects multiple physiological systems in mammals, comparative information from birds will be valuable in the identification of biologically active analogues. Any new functions of leptin discovered in the chicken would be relevant in studies on other vertebrates including fish. To date, leptin has not been cloned from any non-mammalian vertebrates. The structure of avian leptin may therefore provide valuable

clues to the molecular evolution of leptin and to the identification of conserved regions required for its biological actions.

7.3 Potential use

If it is established in poultry that, as in mammals, leptin plays a role in the regulation of food intake, reproduction, and body composition, leptin has potential for commercial application in the poultry industry. First the sequence of chicken leptin protein might be used to design long acting agonists, to reduce appetite. This possibility is particularly relevant to the welfare of the parents of meat type chickens (broiler breeders). Selection for rapid growth rate in these birds results in rapid fat deposition and a large appetite. If adult broilers required for breeding are not subjected to severe food restriction (ie to achieve 40-60% of *ad libitum* fed body mass), reproductive performance is poor (Yu *et al.* 1992). Broiler breeders are consequently always hungry which is perceived to compromise the welfare of the bird. One of the welfare requirements for farm animals in the UK is that they must be able to satisfy hunger and thirst (Webster and Nicols 1988). The administration of leptin or leptin analogues to broiler breeders to suppress appetite could potentially enhance their welfare to the standard required by the Farm Animal Welfare Council (Farm Animal Welfare Council 1988) and simplify management of these birds.

Secondly, knowledge of the sequence encoding the chicken leptin gene might be of value for marker-assisted selection for or against leptin-dependent production traits, such as fat deposition, reproductive function or muscle and bone development. Selection based on phenotype has been the only method of choice until recently for the genetic improvement of farm animals. With the advent of a genome maps from poultry (Burt *et al.* 1995; Smith and Burt 1998; Smith *et al.* 2000) and the development of appropriate statistical methods and high throughput genotyping, systematic analysis of the nature of genetic variation of economically important traits has become a feasible task. This allows the poultry breeder to opt for a marker-assisted selection and marker-assisted introgression in order to accomplish a genetic improvement of poultry stock. Selection on molecular markers or

production trait genes enables the breeder to improve traits such as body composition, which are currently difficult to select for. Regions of the genome (quantitative trait locus, QTL) influencing body composition have been identified (D. Burt, Roslin Institute, unpublished). However, no genes at QTL have been identified in the chicken. Additionally it may be possible to identify polymorphism in the leptin gene in a population of pedigree chicken used for selection purposes. A leptin gene associated polymorphism is likely to be a good candidate for marker-assisted selection for leptin-dependent production traits. This candidate gene approach to selection for production traits has been shown to be successful in pigs (Rothschild and Soller 1997).

In conclusion, the initial objective of this thesis appeared a relatively simple task given the published information on the mouse leptin gene and associated Zooblot information showing hybridization of the gene to chicken gDNA. The failure to clone the gene has highlighted the pitfalls of heterologous RT -PCR cloning, particularly in the hands of scientists who do not have a fundamental understanding of molecular biology. It also illustrates the non-trivial nature of attempting to clone genes from cDNA and gDNA libraries using probes with low homology. The paper arising out of the work described in this thesis has triggered substantial controversy. Major research programmes on the biology and commercial applications of chicken leptin have been initiated in France and the USA based on the assumption that the nucleotide sequence of the chicken leptin cDNA is 95% homologous to the mouse leptin cDNA. The work described in this thesis predicts that the outcomes of these research programmes are likely to be inconclusive.

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APPENDIX

Calculation of incorporation and specific radioactivity

Basic Information (data):

[Brown,T.A. (1998); Sambrook *et al.* 1989; Ausubel *et al.* 1998]

A.

Molecular weight of dATP	= 491.2
Molecular weight of dCTP	= 467.2
Molecular weight of dGTP	= 507.2
Molecular weight of dTTP	= 482.2

Molecular weight of dNTP (average)	= 486.95
	~ 487

B.

1 Becquerel (Bq)	= 1 disintegration per second (d.p.s.)
[the SI unit of radioactivity]	[The number of atoms disintegrating per second]
ie.	= 60 disintegration per minute (d.p.m.)

1 Mega Becquerel (MBq)	= 60×10^6 d.p.m.
ie.	= 6×10^7 d.p.m.

1 Tega Becquerel (TBq)	= 10^6 MBq
ie.	= 6×10^{13} d.p.m.

Count per minute (c.p.m.) = d.p.m. × Counting efficiency.
[The number of γ particles detected per minute]

Counting efficiency for ^{32}P	= 1.
So, in case of ^{32}P, c.p.m.	= d.p.m.

Molar (M)	= Molecular weight in g/L (concentration)
milli Molar (mM)	= Molecular weight in mg/L (concentration)
mole (m)	= Molecular weight in g (absolute weight)
milli mole (mmol)	= Molecular weight in mg (absolute weight)

Specific radioactivity = c.p.m./µg of the substance
[The radioactivity of an element per unit mass]   [probe nucleotide in this instance]

C.

Specifications of radioactive dCTP (Amersham International plc, Buckinghamshire, UK.)
used for labelling the probe nucleotide:

Rate of radioactivity in the dCTP solution   = 37MBq/100µl
ie.   = 0.37MBq/µl.

Specific activity of the dCTP   = 220TBq/mmol.

Calculation:

(Based on Valdecasas, J.C, 1995)

(a) The *amount of radiolabelled dNTP* in the reaction is calculated as follows
[see the basic data given above]:

Volume of dCTP added in one reacton (50µl)= 5µl.
Radioactivity in 5µl dCTP $= 5 \times 0.37$ MBq.
Specific activity of dCTP   = 220 TBq/mmol
ie. $= 2.2 \times 10^2$ TBq/mmol.
ie. $= 2.2 \times 10^8$ MBq/mmol.

So, the amount of dCTP
added in one reaction (50µl)
$$= \frac{5 \times 0.37 \text{ MBq}}{2.2 \times 10^8 \text{ MBq/mmol.}}$$

ie. $= 8.409 \times 10^{-7}$ mmol.

(b) The *incorporation efficiency* measures the amount of radioactive precursor incorporated
into the probe, and is calculated by the formula:

Incorporation efficiency (%) $\quad = \dfrac{\text{Probe c.p.m.} \times 100}{\text{Total c.p.m. in the reaction}}$

[Usually, this value was ranging from 8-11% with an approximate average of 10%.]

(c) *Theoretical yield* of probe is the calculated amount of probe DNA sequence generated.

So, assuming that

(1) the four nucleotides are evenly distributed in the DNA sequence used as template to make the probe , and
(2) an average dNTP has a molecular weight of 487 (see the basic data given above)

the theoretical yield of probe nucleotide is given by the following formula:

Theoretical yield (mg) $\quad = 8.409 \times 10^{-7}\% \; 87\% \; 4\% \text{ inc. efficiency (\%)}$
ie. $\quad = 1.64 \times 10^{-3}\% \text{ inc. efficiency (\%)}$

Theoretical yield (µg) $\quad = 1.64 \times \text{inc. efficiency (\%)}$

(d) Since 0.5µl of the(phenol-chloroform precipitated and sephadex column-spun) probe solution was diluted in 99.5µl of MQ water and 10µl of such dilute probe solution was mixed with the cocktail fluid for noting the radioactivity count in the γ-counter, the dilution factor is calculated as:

Dilution factor $\quad = \dfrac{100 \times 50}{10 \times 0.5}$

ie. $\quad = 1000$

ie. $\quad = 10^3$

(e) The *specific activity* of the probe, defined as the radioactivity per unit mass, is calculated as:

Specific activity (dpm/µg) $\quad = \dfrac{\text{Scint. count} \times \text{Dil. factor}}{\text{Theoretical yield (µg)}}$

Calculation of Tm

According to Meinkoth and Wahl (1984)

Tm = $81.5°C + 16.6 (\log M) + 0.41 (\%G+C) - 0.61(\%form.) - 500/L$

{where M is the monovalent cation concentration, (%G+C) is the percentage of G and C nucleotides in the DNA and L is the length of the duplex in base pairs. The relationship holds for DNAs with (G+C) contents of 30-75%.}

So, Tm = $81.5°C + 21.06609195403 + 16.6 (\log M)$
= $102.566091954 + 16.6 (\log M)$

Molarity at 0.4 = 0.066
Molarity at 0.2 = 0.033
Molarity at 0.1 = 0.016

For 0.4 × SSC, Tm = $102.566091954°C + 16.6 \% -1.180456064458$
= $102.566091954°C + -19.59557067$
= $82.97052128403°C$
~ 83°C

For 0.2 × SSC,Tm = $102.566091954°C + 16.6 \log 0.033$
= $102.566091954°C + 16.6 \% -1.481486060122$
= $102.566091954°C + -24.59266859803$
= $77.97342335597°C$
= 78 °C

For 0.1 × SSC, Tm = $102.566091954 °C + 16.6 \log 0.016$
= $102.566091954 °C + 16.6 \% -1.795880017344$
= $102.566091954 °C + -29.81160828791$
= 72.75448366609
~ 73°C

Hybridization conditions at 50mM Sodium phosphate

$\log \{[Na]/(1+0.7[Na])\}$ = $\log \{0.05/[1+(0.7 \% 0.05)]\}$
= $\log \{ 0.05/[1+ 0.035]\}$
= $\log \{ 0.05/1.035\}$
= $\log 0.04830917874396$
$\log 0.04830917874396$ = -1.315970345457

16.6 × -1.315970345457   = -21.84510773458
So, Tm   = 102.566091954 °C + -21.84510773458
    = 80.72098421942
    ~ 81°C

So, Tm - 20 (ideal for perfect match) = 61°C.
Tm-25 (ideal for imperfect match)   = 56°C.
Actual hybridization carried out   = 57°C.

Tm   = 81.5°C + 16.6 (logM) + 0.41 (%G+C) - 0.61(%form.) - 500/L
At 2 × SSC (lowest stringency
Washing starts)   = 102.6°C + 16.6 logM
ie.   = 102.6°C + 16.6 % log 0.33
ie.   = 102.6°C + 16.6 % -0.4814860601221
ie.   = 102.6°C + ⁻7.992668598027
ie.   = 94.60733140197°C
ie.   = 95°C.
Tm - 25°C   = 70°C.

Calculation of the mismatch in the chicken leptin-like sequence:

From Anderson, 1999,

Tm   = 81.5°C+ 16.6 × log ([Na]/1+0.7[Na]) +0.41 (% G+C) - 500/L - P - (0.63 × % formamide)

{where Na⁺ is the monovalent cation concentration, (%G+C) is the percentage of G and C nucleotides in the DNA and L is the length of the duplex in base pairs. P is the percent mismatching. The relationship holds for DNAs with (G+C) contents of 30-75%.}

The probe is 348bp long between primers RF2 and RR1, and

(% G+C) is estimated to be
 76G + 115C   = 191/348 × 100
    = 54.88505747126

0.41 (% G+C)   = 22.50287356322

500/L   = 1.436781609195

0.41 (%G+C) -500/L   = 21.06609195403

So, Tm
$= 81.5°C + 16.6 \times \log ([Na]/1+0.7[Na]) + 21.06609195403 -P$

Tm
$= 81.5°C + 16.6 \% \log ([Na]/1+0.7[Na]) - P.$

Tm
$= 102.566091954 + 16.6 \times \log ([Na]/1+0.7[Na]) - P$

Calculate Tm at $0.4 \times$, $0.2 \times$ and $0.1 \times$ SSC.
Molarity at $0.4 \times$ SSC $= 0.066$
Molarity at $0.2 \times$ SSC $= 0.033$
Molarity at $0.1 \times$ SSC $= 0.016$

For Molarity at $0.4 \times$ SSC,
$([Na]/1+0.7[Na])$ $= 0.066/[1+ 0.7 \% 0.066]$
    $= 0.066/ [1+ 0.0462]$
    $= 0.066/1.0462$
    $= 0.06308545211241$
Log 0.06308545211241 $= -1.20007078015$
$16.6 \times -1.20007078015$ $= -19.92117495048$

$102.566091954 + ^{-}19.92117495048$
    $= 82.64491700355$
ie. $\sim 83°C - P$

For Molarity at $0.2 \times$ SSC,
$([Na]/1+0.7[Na])$ $= 0.033/ [1+ 0.7 \% 0.033]$
    $= 0.033/[1+ 0.0231]$
    $= 0.033/1.0231$
    $= 0.03225491154335$
Log 0.03225491154335 $= -1.491404144788$
$16.6 \times -1.491404144788$ $= -24.75730880348$
$102.566091954 + -24.75730880348$
    $= 77.80878315052$
    $\sim 78°C -P$

For Molarity at $0.1 \times$SSC,
$([Na]/1+0.7[Na])$ $= 0.016/ [1+ 0.7 \% 0.016]$
    $= 0.016/[1.0112]$
    $= 0.01582278481013$
Log 0.01582278481013 $= -1.800717078282$
$16.6 \times -1.800717078282$ $= -29.89190349949$
$102.566091954 + -29.89190349949$
    $= 72.67418845451$
    $\sim 73°C -P$

From table 4.2, it is obtained that the sheep leptin sequence hybridizing with the probe (with a 17% mismatch) disappeared at a temperature of 66°C at a Tm + P of 73°C whereas the chicken leptin-like sequence disappeared at 66°C at a Tm + P of 78°C.

So, the mismatch of the chicken leptin-like sequence = 78 – 73 = 5.

In other words, the percentage similarity of the chicken leptin-like sequence with the mouse leptin probe is 83-5 = 78. Considering the entire length including the non-coding regions, it must be less than 70%.

Note:

Note: Ideally, for perfectly matched hybrids, hybridization should be carried out at a temperature for which the rate is maximal, i.e. 20-25°C below the Tm for DNA:DNA hybridizations and for imperfectly matched hybrids, the temperature of hybridization and washing solutions are lowered to stabilize the hybrids (Sambrook et al, 1989; Wetmur, 1991; Anderson, 1999). These equations are derived from solution hybridization studies, but take into account the higher salt concentrations appropriate for filter hybridization. However, they give only a rough estimate of the Tm for filter hybridizations. As a consequence of binding nucleic acid to filters, the Tm of hybrids is often lower than would be predicted from solution hybridization studies (Beltz et al, 1983). However, filter-bound hybrids do not dissociate as quickly as hybrids in solution (Church and Gilbert, 1984), so this must be taken into account when planning times of washing (Anderson, 1999).