Detection of Trait-Associated Restriction Fragment Length Polymorphisms in Chicken

by

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Abstract

The gene encoding chicken growth hormone (GH) was isolated from a chicken genomic library. The size of the gene was 4 kb. It was digested with PstI and subcloned into pUC18. Three of the PstI fragments were used for restriction fragment length polymorphisms (RFLPs) analysis at the GH locus in two chicken strains (fat and lean line). Four polymorphic sites were detected using a PstI fragment (PII) as a probe. One polymorphism was located at a SacI restriction site (PS1), and three at MspI sites (PM1, PM2 and PM3). A method based on polymerase chain reaction (PCR) was developed for detecting polymorphisms at PM3 site. A fragment of 823 base pairs which contained the PM3 polymorphic site was amplified. Three genotypes (+/+, -/- and +/-) were distinguished by examining the MspI digested PCR products in either agarose or polyacrylamide gel.

Ten anonymous cDNA clones were also isolated from a chicken liver cDNA library and used for RFLPs analysis. Three of these clones were found to be able to detected RFLPs at MspI sites in chicken strains (strain 7, 8, 9, 8R, S and K) indicating that a high frequency of genes are polymorphic and can be used as markers in mapping experiments. One of the three clones was present on a haploid genetic element. Segregation analysis showed that the inheritance of this haploid gene was determined by the genotype of the female parent.

Résumé

Le gène de l'hormone de croissance (GH) a été isolé à partir d'une librairie génomique de poulet. La taille du gène est de 4 kb. Il a été digéré avec PstI et cloné dans pUC 18. Trois fragments PstI ont été utilisés pour l'analyse du "Polymorphismes de Longueur de Fragments de Restriction" (PFLR's) au niveau du locus GH dans deux lignées de poulets (gras et maigre). Quatre sites polymorphiques ont été détectés à l'aide d'un fragment PstI (PII) utilisé comme sonde. Un polymorphisme a été localisé sur un site de restriction SacI (PS1), et trois sur des sites MspI (PM1, PM2 et PM3). Une méthode basée sur la réaction de polymérisation en chaîne (PCR) a été développée pour détecter les polymorphismes au site PM3. Un fragment de 823 bases contenant le site polymorphique PM3 a été amplifié. Après avoir digéré les produits de PCR avec MspI et les avoir séparés par electrophorèse sur des gels d'agarose ou de polyacrylamide, trois génotypes (+/+ i-l-j+/-1) ont pu être déterminés.

Aussi, dix clones provenants d'une librairie d'ADNc de foie de poulet ont été utilisés pour l'analyse par RFLP. Trois de ces clones possèdent des RFLP's à des sites MspI dans certaines lignées de poulets (lignées 7, 8, 9, 8R, S & K). Ceci indique qu' un grand nombre de gènes sont polymorphiques et peuvent être utilisés comme marqueurs pour faire du "mapping". Un des trois clones se comporte comme un élément génétique haploide et une analyse de ségrégation a montré que l'héritabilité de ce gène haploide était déterminée par le génotype du parent femelle.

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I. Introduction

Restriction fragment length polymorphisms (RFLPs) have been widely used as genetic markers since the initial discovery of RFLPs associated with human β- and τ- globin loci. Many examples of RFLPs detected by human gene probes or randomly cloned DNA segments have been reported. Analysis of RFLPs became an essential tool to map single genes (Botstein *et al.*, 1980), to diagnose genetic diseases (Gusella *et al.*, 1983) and to identify markers associated with variations in complex genetic traits (Lander and Botstein, 1989; Paterson *et al.*, 1988; Soller and Beckman, 1983). RFLPs as genetic markers have their particular usefulness in genetic improvement programs of plants and domestic animals. The advantage of using this kind of genetic markers is that they lack dominance, exist in multiple allelic forms, do not have pleiotropic effects on other characters and do not require expression for their detection. Landegren *et al.* (1988) and Tanksley *et al.* (1989) reported the application of RFLPs to plant breeding practices. Soller and Beckman (1986) and Kuhnlein *et al.* (1989, 1990b) discussed the application of RFLP analysis to poultry breeding. RFLPs can be detected by using clones of specific genes or random DNA segments as probes (Cooper and Schmidtke, 1984).

It is well known that chicken growth hormone (GH) influences a number of distinct aspects of the physiology of chickens, including growth, metabolism of lipids, carbohydrates and proteins and endocrine secretion (Scanes *et al.*, 1983). It is also involved in the regulation of the immune system (Gala, 1991).

RFLPs in the GH gene of chickens were identified by using the turkey GH cDNA

gene as a probe (Fotouhi *et al.*, 1993). Four polymorphic sites were found at SacI and MspI restriction sites, respectively. These polymorphisms can potentially be used as genetic markers to select for abdominal fat content or for resistance to avian leukosis virus (ALV) and Marek's disease (MD).

The full potential usefulness of RFLPs in poultry breeding is limited by the paucity of presently available markers. Furthermore, the traditional Southern blotting method for RFLP detection is too labour intensive for determining genotypes in a large breeding population. In the present study we planned to identify more genetic markers and to develop a rapid assay for the detection of the GH-RFLPs known to be associated with economically important traits.

The objectives of this study were: (1) to isolate the GH gene from a chicken genomic library; (2) to sequence the gene and locate the exact position of the known GH gene polymorphisms (PS1, PM1, PM2 and PM3); (3) to develop a PCR-based method to detect these polymorphisms; and (4) to isolate other polymorphic cDNA clones which can be used as genetic markers for mapping specific production traits in chickens. The first part of the thesis is focused on the first three objectives. In the second part, random cDNA clones were screened for polymorphisms. Three out of ten clones were found to be polymorphic. They were tested for co-selection with disease resistance and for correlation with two production traits, the housing body weight (HBW) and the age at first egg (AFE).

II. Literature Review

1. Growth hormone and growth hormone gene

1.1. Growth hormone

Growth hormone (GH) is a protein hormone produced by the somatotrophs of the anterior pituitary gland of birds and other vertebrates. The secretion of GH in birds is under hypothalamic control and involves three peptidergic releasing factors, namely the growth hormone-releasing factor (GRF) (stimulatory), the thyrotropin-releasing hormone (TRH) (stimulatory) and somatostatin (SRIF) (inhibitory) (Scanes and Lauterio, 1984).

1.2. Growth Hormone and Growth

The hormone control of growth in poultry and other species is complex. The available evidence supports the concept that growth hormone and the thyroid hormones are the principal hormones responsible for the attainment of normal growth in the domestic fowl (Scanes *et al.*, 1983). The plasma GH concentration varies during the development and growth. It is high in the rapid growth period of early age and low in later stages of development and in the adult. This change of plasma GH concentration with age has been observed in the domestic fowl (reviewed by Scanes and Harvey, 1982), turkeys (Harvey *et al.*, 1977; Proudman and Wentworth, 1980) and ducks (Harvey and Phillips, 1980). It may be pertinent that neonatal plasma concentrations of GH are higher in faster growing broiler strains of chickens

than in slower growing "laying type" strains (Scanes and Harvey, 1982). Furthermore, the plasma concentration of GH declines with age earlier in "broiler" than in "laying type" strains (Scanes and Harvey, 1982), and earlier in "broiler strains" selected for growth than in random-bred lines (Burke and Marks, 1982).

The ability of mammalian GH to affect the growth and metabolism of birds is controversial. In early studies with relatively impure GH preparations, no effect of ovine or bovine GH was observed on the growth rate of intact chickens (Libby *et al.*, 1955; Glick, 1960). Using purer bovine GH preparations, Tojo *et al.* (1978, 1979) similarly found no effects of mammalian GH on the growth of intact chicks or on the epiphyseal cartilage of hypophysectomized chicks. However, the administration of porcine GH to hypophysectomized chicks markedly stimulates body and skeletal growth (Scanes *et al.*, 1984).

The growth response of transgenic mice appears to be independent of the species specificity of the coding sequence of the growth hormone gene, since bovine, human or porcine growth hormone coding sequence fused to the metallothionein promoter give essentially similar increases in growth rate (Palmiter *et al.*, 1982, 1983; Michalska *et al.*, 1986a). For domestic animals, however, the coding sequence may need to encode the hormone specific to each species, since transgenic pigs expressing the human or the bovine growth hormone did not show any increase in growth rate (Hammer *et al.*, 1985; Pursel *et al.*, 1986), whereas over-expression of the porcine coding sequence led to an increase by about 25% (Michalska *et al.*, 1986b).

1.3. Growth hormone and other production traits

In addition to a major impact on growth, GH plays a role in mammary gland development and lactation in dairy cattle. Further, it was shown that cells involved in immunity have specific receptors for GH and that bovine GH (in mouse cell culture) or human GH (in human cell culture) significantly stimulated erythroid colony formation (Golde and Bersch, 1977). GH is also involved in the regulation of the immune system (Gala, 1991). Marsh *et al.* (1984) investigated the effect of bovine GH administration to sex-linked dwarf chicks which show distinct abnormalities of the immune system with a poor primary antibody response and a low weight bursa of Fabricius and thymus. Treatment with GH was found to increase the primary antibody response and the bursal weight. Glick (1960) also reported that administration of bovine GH increased the weight of the bursa of Fabricius.

According to Scanes *et al.* (1983), GH also affects intermediary metabolism in chickens and effects on lipid, carbohydrate and protein metabolism have been documented. It is thought to act on lipid metabolism by stimulating lipolysis, inhibiting lipogenesis in the liver, and increasing glucose uptake by adipose tissue (Scanes and Harvey, 1982; Rudas and Scanes, 1983). Studies provide strong support that GH is the pituitary hormone responsible for some of the effects observed following hypophysectomy. For instance, administration of GH increases lipolysis by chicken, turkey, and hypophysectomized pigeon (Harvey *et al.*, 1977). Similar effects of GH have been observed in hypophysectomized ducks from *in vivo* studies. Bovine GH has been found to increase the concentration in the circulation of fatty acids, glucose and

amino acids, and it also acts to increase the release of insulin and decrease that of glucagon (Foltzer *et al.*, 1975; Foltzer and Miahle, 1976).

1.4. The structure of growth hormone gene

Growth hormone (GH), prolactin (PRL) and chorionic somatomammotropin (CS, placental lactogen) are a family of polypeptide hormones. They are related by function, immunochemistry, and structure. They have similar sizes (190 to 199 amino acids among various species) and protein structures (Miller and Eberhardt, 1983).

In 1977, Seeburg and coworkers first isolated and characterized the rat GH cDNA. Thereafter the human (Roskam *et al.*, 1979), bovine, porcine (Woychick *et al.*, 1982; Seeburg *et al.*, 1983), ovine (Byrne *et al.*, 1987), and turkey (Foster *et al.*, 1990) GH cDNA clones have been isolated and characterized.

The chicken GH has a molecular weight of 23 KDa and contains 191 amino acids (Scanes, 1987). The gene is located on the long arm of the chromosome 1 (Shaw *et al.*, 1988). The chicken cDNA sequence has been reported by Lamb *et al* (1988). It encodes a 25 amino acid amino terminal precursor segment and a 191 amino acid mature apoprotein segment. The cDNA sequence of chicken GH has 70% to 78% homology with other mammalian GH cDNAs (Queen and Korn, 1984). Chicken and duck GH cDNAs show 92% homology throughout the coding region and 90% homology in the 5' untranslated regions (Chen *et al.*, 1988).

The genomic sequence of chicken GH was reported by Tanaka *et al.* (1992) while our study was in progress. Their sequence included 500 bp of the 5'-flanking region.

The chicken GH gene consists of five exons and four introns as has been observed in the mammalian GH gene. It is about 3.5 kb in size. The promoter region of chicken GH gene has no overall homology with those of mammalian GH genes, but contains a short sequence which is highly homologous to the antisense strand sequence of the proximal GHF-1/pit-1 binding site in the promoter region of the mammalian GH gene (Seeburg *et al.*, 1977).

2. Restriction fragment length polymorphisms (RFLPs) and animal breeding

2.1. RFLPs as genetic markers:

Genetic maps comprising closely-spaced DNA markers are useful for genome analysis. DNA markers that are shown to be genetically linked to a trait of interest can be used in gene cloning, medical diagnostics, and for trait introgression in plant and animal breeding programs (Landegren *et al.*, 1988; Tanksley *et al.*, 1989). Clues to the location of gene come from comparing the inheritance of a trait with the inheritance of markers of known chromosomal location. Coinheritance or genetic linkage of disease gene and marker indicates that they are physically close together on the chromosome (White and Lalouel, 1988). A marker must be polymorphic, that is it must exist in different forms so that the chromosome carrying the mutant gene can be distinguished from the chromosome with the normal gene by the form of the marker it also carries (Botstein *et al.*, 1980; Wyman and White, 1980).

RFLPs constitute markers for alleles at a particular locus. When a mutation occurs in a restriction cutting site of a specific gene owing to base change, deletion,

insertion or rearrangement, the length of fragments after digestion of genomic DNA with restriction enzymes will change. The steps for detecting RFLPs are digestion of the genomic DNA with a restriction enzyme, separation of the DNA fragments in a agarose gel, transfer of the DNA from the agarose gel to a membrane, hybridization of the membrane with the radio-labelled DNA segment of interest and autoradiography of the hybridized membrane using an X-ray film.

The first RFLPs were reported by Arber and Kuhnlein (1967). Since then many examples of RFLPs detected by human gene probes or random cloned DNA segments have been reported (Cooper and Schmidtke, 1984). RFLPs can be more efficiently detected using long probes to screen multiple restriction sites at a given locus (Litt and White, 1985) and by using restriction endonuclease such as TaqI or MspI which cleave at sequences containing the highly mutable CpG doublet (Barker *et al.*, 1984).

RFLPs have been extensively used to search for markers linked to disease loci whose gene product is unknown (Gusella., 1983). RFLPs closely linked to, or within disease loci can be used for antenatal diagnosis, carrier detection and counselling, provided that sufficient pedigree data exist for a given family to establish linkage between marker alleles and the disease (Weatherall, 1985). RFLPs are also used in population genetics. As an example the study of RFLPs associated with haplotypes led to the discovery of meiotic recombination hot-spots in gene clusters (Chakravarti *et al.*, 1984).

2.2. RFLPs and animal breeding:

RFLPs have been used in animal breeding especially since the discovery of hypervariable loci (minisatellites and microsatellites; Bell *et al.*, 1982; Goodbourn *et al.*, 1983; Jeffreys *et al.*, 1985a; Nakamura *et al.*, 1987; Simmler *et al.*, 1987). There are many possible applications of RFLP analysis in animal breeding. For example identification of stolen animals, verification of semen samples used in artificial insemination, determination of pedigree, linkage analysis and the search for quantitative trait loci of economic importance (Beckmann and Soller, 1983; Soller and Beckmann, 1983). Parent-offspring testing in the wild could lead to a more detailed understanding of the genetic structure of natural populations, and could also be used to maximize outbreeding in captive colonies of endangered species.

Soller and Beckman (1986) discussed the application of RFLP analysis in poultry breeding. In chickens, Kuhnlein *et al.* (1989) used RFLP analysis to gain evidence whether endogenous virus (*ev*) genes affect production traits in White Leghorns. Further, the analysis of RFLPs have been applied by Kuhnlein *et al.* (1990b) for determining inbreeding coefficients in strains of chickens, which may be useful in estimating the likelihood of response to selection. Hillel *et al.* (1989) used human minisatellite probes which cross-hybridized to DNA of several species of poultry (chicken, duck, turkey and goose), and detected a high frequency of polymorphisms. The resulting RFLPs are individual specific, and allow the discrimination even between closely related birds. The pattern of poultry DNA fingerprinting is different from that of humans and other animals having a higher average proportion of large

DNA fragments. Uni *et al.* (1992) reported a study utilizing RFLP analysis to identify the MHC Class IV genotypes in randomly selected chickens from a commercial meat-type grandparent line. A high frequency of polymorphisms was observed following digestion with each of the two restriction enzyme PvuII and BglI. Dunnigton *et al.* (1990) analyzed DNA fingerprints of chickens which were selected for high and low body weight for 31 generations by using Jeffreys' minisatellite probe 33.6.

Montgomery's study (1992) also showed that sheep and cattle cDNA probes, including candidate genes for production traits, revealed a high frequency of RFLPs suitable for genetic mapping in sheep.

2.3. RFLP of growth hormone gene:

GH gene polymorphisms have been found in many different animals and in some cases have been associated with production traits. Nielsen and Larsen (1991) reported RFLPs of the GH gene in pigs. The polymorphic sites were detected with the restriction enzymes DraI and TaqI. A comparison of Danish pig populations showed significant differences among the frequencies of the different alleles. In addition, HaeII and MspI polymorphisms in the second intron of the porcine growth hormone gene were identified by Kirkpatrick (1991). RFLPs at the ovine locus for growth hormone were observed by using TaqI and PvuII. Both RFLPs are controlled by three co-dominant and one recessive null allele as determined from data on a large number of sire-dam-offspring combinations from two merino flocks, Medium Peppin and Medium Non-Peppin (Parsons *et al.*, 1992).

GH gene polymorphisms at MspI sites have been found to be co-selected with selection for milk fat content in lines of cattle (Hoj *et al.*, 1993). In chickens, Fotouhi *et al.* (1993) identified GH DNA polymorphisms which respond to divergent selection for abdominal fat content in broiler chickens. In their study, four DNA polymorphisms were observed by using Southern blotting, one at a SacI restriction site and three at MspI restriction sites.

2.4. Improvement of RFLP detection:

The traditional DNA Southern blot has been the method most commonly used for detecting RFLPs (Southern, 1975). The basic steps involved in demonstrating RFLPs include: (i) DNA extraction, (ii) preparation of probes and (iii) DNA analysis, which includes digestion of DNA with restriction enzymes, electrophoresis and blotting, followed by hybridization and autoradiography of the blots.

Little information is as yet available on the average number of probes and restriction enzymes that need to be tested in order to detect a useful polymorphism, where a useful polymorphism is defined as one for which allelic frequencies are in the range 0.1 to 0.9 (Beckmann and Soller, 1983). Jeffreys (1979) detected two useful and one rare polymorphism in human DNA using a β-globin cDNA probe and eight restriction enzymes. Wyman and White (1980) tested five randomly chosen unique DNA probes and two restriction enzymes against human DNA. One of the probes uncovered a locus showing a high degree of polymorphism with both enzymes. Rom (1982) found polymorphism in DNA from four strains of tomato digested with a

single enzyme and tested against a single random DNA probe. The frequency of detected polymorphisms per probe x enzyme combination tested can be expected to vary widely between species and populations. Nevertheless, on the basis of the above results, it seems justifiable to assume that a range of 10-20 probe x enzyme combinations have to be tested to provide an useful polymorphism (Beckmann and Soller, 1983).

Although Southern blotting is and will be the main approach to search for new RFLP as genetic markers, the disadvantage of Southern blotting (including DNA fingerprinting) for detecting a known RFLP is that it is time-consuming and laborious, especially when a large number of samples are dealt with (Beckmann and Soller, 1983). Recently a new technique was developed which provides another possibility to rapidly analyze homologous segments from a large number of individuals. This technique called "**polymerase chain reaction**" (PCR) is utilized to amplify a specific DNA sequence for its subsequent cloning, sequencing or analysis by restriction enzymes (Erlich and Arnheim, 1992). There are a number of advantages to using a PCR assay for determining the genotype of a DNA sample for a known RFLP. The assay takes only a few hours compared to Southern blotting (Southern, 1975), which can take days or weeks. Further, PCR can be automated and thus a large number of samples can be rapidly typed with little labour. Furthermore, only picograms or nanograms of DNA are needed for PCR, whereas micrograms are needed for Southern blotting of genomic DNA. In one PCR reaction, many distinct genetic loci can be typed at the same time by multiplex amplification using multiple primer pairs. As a

result of these economies, a number of laboratories have established PCR assays for known RFLP markers and many searches for new polymorphisms have focused on those that can be assayed by PCR.

3. Polymerase Chain Reaction (PCR) and its application to RFLPs detection

3.1. PCR technology:

In 1984, a team of scientists at Cetus Corporation developed a DNA amplification procedure based on an *in vitro* rather than an *in vivo* process, known as the **polymerase chain reaction** (PCR) (Saiki *et al.*, 1985; Mullis *et al.*, 1987). This method can produce large amounts of a specific DNA fragment from a complex DNA template in a simple enzymatic reaction. The amount of DNA needed for PCR (Fig. 1) is less than a microgram of total genomic DNA, and can be as little as a single DNA molecule. The two oligonucleotide primers which provide the starting points for DNA synthesis, DNA polymerase, and a mixture of all four deoxynucleotide precursors are added to a tube containing the DNA. Each cycle of reaction includes three steps, primer annealing, DNA synthesis and DNA denaturation. About 30 to 60 cycles are usually needed (Innis *et al.*, 1988). The temperature in each step of a cycle is important and requires that the reaction is carried out in a thermal cycler with very accurate temperature control.

The PCR is an extraordinarily powerful tool for the analysis of genes. It is used for cloning genes (Paabo *et al.*, 1989), for *in vitro* mutagenesis (Higuchi *et al.*, 1988), for mapping and sequencing large genomes (Wong *et al.*, 1987; Innis *et al.*, 1988), and

for diagnosing human inherited disorders (Laure *et al.*, 1988; Brisson-Noel *et al.*, 1989). The speed, specificity, and sensitivity of PCR has revolutionized molecular genetics.

3.2. PCR-based method for detection of RFLPs:

The most basic requirement for detecting RFLPs by PCR analysis is a set of primers that flank the specific polymorphic DNA region. Once the primers have been designed and the PCR product amplified, various procedures can be used to determine the genotype of the amplified DNA segment. Direct DNA sequencing can be used, which is the most comprehensive way of revealing polymorphisms. The sequence analysis of amplified DNA was initially performed on cloned PCR products (Scharf *et al.*, 1986), but the increase in specificity of PCR when thermostable DNA polymerase is used, has made direct sequencing of PCR products the preferred approach for most applications.

An alterative approach is based on gel electrophoretic methods. Sequence differences can be detected by running the two DNA samples in adjacent lanes of an acrylamide gel that has a gradient of a DNA denaturant along the axis of DNA migration (Fisher *et al.*, 1983).

Another approach is called PCR/SSO (sequence-specific oligonucleotide) typing. Short labelled oligonucleotide probes (allele-specific probes) are used to distinguish single nucleotide differences in immobilized PCR products (the dot blot method) by ensuring through appropriate hybridization conditions that the probe will only bind

to completely complementary target sequences (Saiki *et al.*, 1986).

The PCR reaction can also serve to recognize sequence variants occurring at the 3' end of one of the primers. A primer mismatched at its 3' end with the template strand is much less efficiently extended by DNA polymerase (particularly if it lacks the 3'-5' exonuclease activity) than a completely complementary primer. This approach is termed allele-specific amplification (Wu *et al.*, 1989).

A new technique called single-stranded conformation polymorphism (SSCP) analysis is especially useful (Watson *et al.*, 1992). A single nucleotide difference between two short single-stranded DNA molecules induces a difference in the conformation adopted by the two strands following denaturation. Remarkably, the conformational difference is sufficient to produce changes in the molecules' electrophoretic mobilities on neutral polyacrylamide gels. The method was developed by using genomic DNA fragments produced by digestion with restriction enzymes, but it has now been simplified by using PCR to amplify those regions of a gene that may contain mutations.

RFLPs can also be detected by the oligonucleotide ligation assay (OLA) (Landegren *et al.*, 1988). This recently developed assay is capable of detecting single base differences and is based on the usage of the enzyme DNA ligase. In this procedure, an amplified sample is divided into two aliquot. Two 20-nucleotide long probe oligonucleotides that abut each another on the same strand are annealed to the denatured PCR product. One is allele-specific (capture probe) while the other serves as a reporter. Successful ligation (captor of the reporter probe) indicates

sequence identity between the allele-specific probe and the PCR product, while absence of ligation indicates the presence of a mutation.

Williams *et al.* (1990) used a PCR based method to detect DNA polymorphisms by arbitrary primers, these polymorphisms are useful as genetic markers, and are called randomly amplified polymorphic DNA (RAPD) markers.

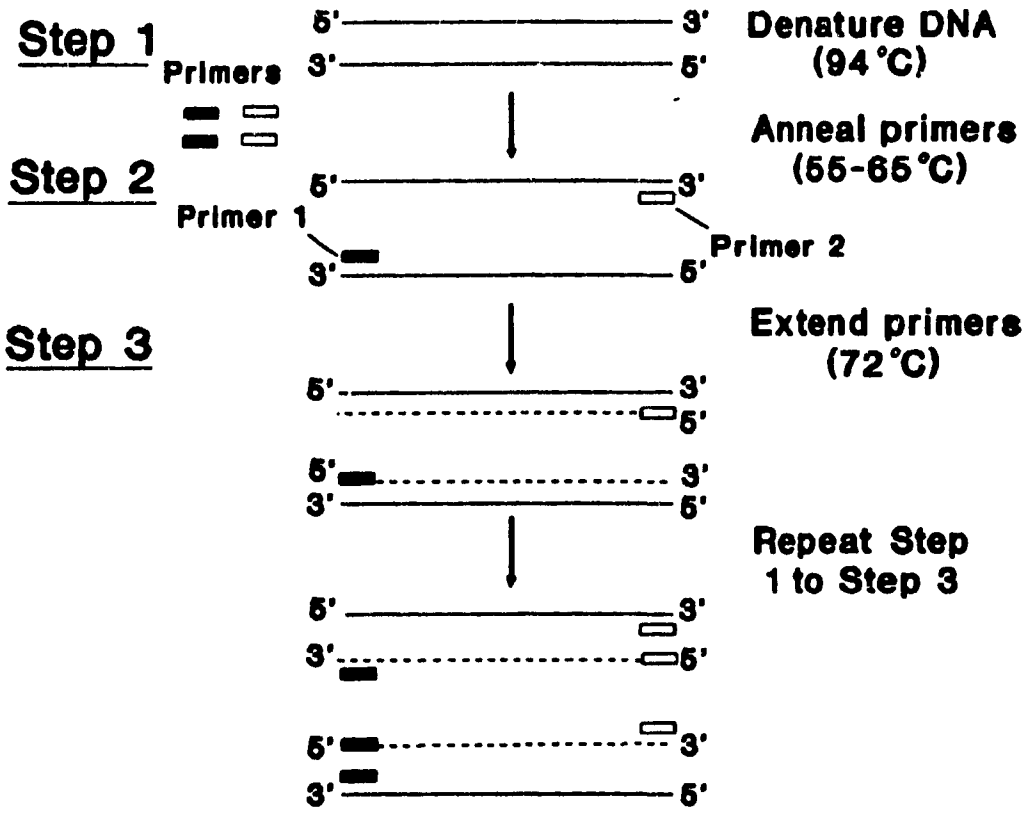

Fig. 1. Polymerase chain reaction procedure.

III. Trait-associated Growth Hormone Gene Polymorphism: Development of a PCR-Based Method for their Detection

1. Materials and Methods:

1.1. Genomic library

The chicken genomic library was purchased from Stratagene. It was made from genomic DNA isolated from blood of eight month old Cornish White Rock chickens. The cosmid vector pWE15 (Fig. 2) was linearized with the restriction enzyme BamHI and the genomic DNA was partially digested with Sau3AI, which creates BamHI compatible ends. DNA fragments in the range of 35 to 41 kb were isolated and ligated to the linearized vector DNA. They form a concatemer of vector and genomic DNA fragments which can be packaged into phage lambda by a packaging extract which recognizes and packages any ligated DNA that carries two *cos sites* 35 to 45 kb apart. These segments of DNA were then introduced into *E. coli* by infection. They become circularized inside the *E. coli* cells and replicate like ordinary drug-resistant plasmids.

1.2. Probe preparation

The probe used to screen the chicken genomic library in this study was the entire turkey GH cDNA (894 base pairs). The turkey GH cDNA gene (tGH) shares 95% homology with the chicken GH sequence (Foster *et al.*, 1990). The tGH sequence (clone pGH-25) was ligated into the EcoRI site of the plasmid pUC13 (Fig. 3) and

was kindly provided by C. Karatzas.

A small-scale isolation (miniprep) of the plasmid pGH-25 was carried out according to Sambrook *et al.* (1989). In brief, bacteria carrying the plasmid pGH-25 were used to inoculate 15 ml of Luria-Bertani culture medium (LB) containing 100 μg/ml ampicillin in a 50 ml tube. Incubation was overnight at 37^{0}C in a water bath with shaking (250 rpm). Luria-Bertani culture medium consists of 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl per litre (pH 7.2). Aliquot of 1.4 ml of overnight culture were centrifuged in 1.5 ml Eppendorf tubes at maximum speed for 20 second in a microcentrifuge. The bacterial pellet was re-suspended in 200 μl of 50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8.0 and kept on ice. Then 300 μl of freshly prepared 0.2 M NaOH, 1% SDS was added to lyse the cells. The protein and bacterial DNA were precipitated by the addition of 200 μl of 3 M potassium acetate, pH 5.2. The precipitate was removed by centrifugation at 13,000 rpm for 8 minutes. The supernatant was extracted once with one volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v) and once with one volume of chloroform-isoamyl alcohol (24:1 v/v), followed by precipitation with 2 volumes of 95% ethanol. The pellet (plasmid DNA) was washed with 70% ethanol repelleted by centrifugation, dried and dissolved in 50 μl of 5 mM Tris-HCl, 0.1 mM EDTA (pH 7.5) per tube. The RNA present in the sample was degraded by incubating the sample with RNase (20 μg/ml) for one hour at room temperature.

The plasmid pGH-25 was digested with restriction enzyme EcoRI in the presence of 2x One-Phor-All buffer (Pharmacia) at 37^{0}C for 2 hours. Following the digestion,

the insert (894 bp) was separated from the vector (2.7 kb) by electrophoresis on a 1% low melting temperature (LMT) agarose gel. The tGH sequence was recovered from the LMT agarose by the procedure described by Sambrook *et al.* (1989). Briefly, the gel slice containing the tGH band was cut out from the gel and placed in an Eppendorf tube. Three ml of double distilled water was added for each gram of gel. The agarose was dissolved by heating at 65^0C for 10 minutes. This preparation was used directly for preparing the probe.

The probe was labelled by the random priming method (Feinberg and Vogelstein, 1983) with α-^{32}P-dCTP using Phamarcia's T_7 Quick Primer/labelling kit. Each reaction mixture was prepared by denaturing 50-100 ng of the probe by boiling for 10 minutes. The reaction was placed on ice, 10 μl reagent mix containing ATP, TTP, GTP and primer sequences, 5 μl of $^{32}\alpha$P-dCTP and 1 μl T_7 DNA polymerase were added and the reaction was incubated at 37^0C for 2-4 hours. In this reaction the primers bind to the ssDNA randomly and the DNA polymerase incorporates the nucleotides including the radioactive dCTP starting from the primers and using the ssDNA as a template. The labelled probe was purified by filtration through a Sephadex column (Nick-column, Pharmacia). The radioactive probe was denatured by boiling for 10 minutes and was chilled on ice.

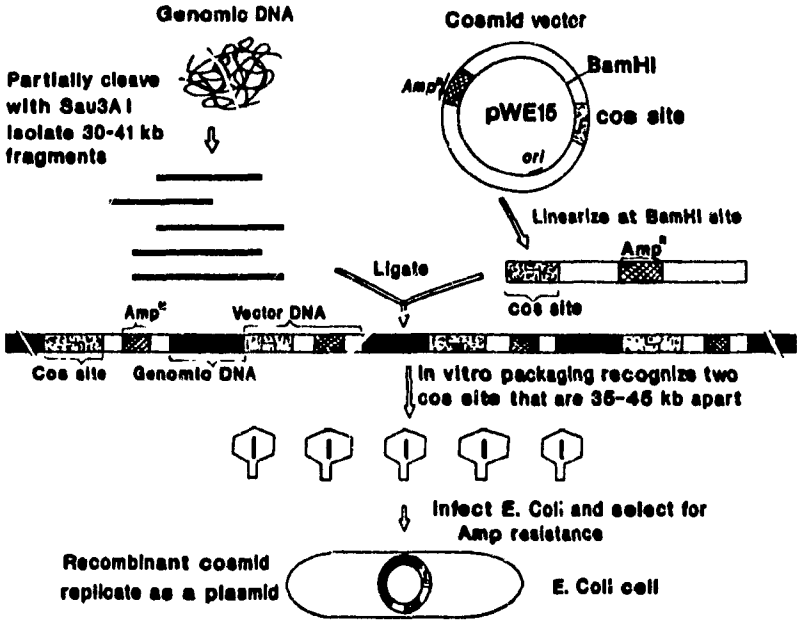

Fig. 2. Construction of the genomic library. The cosmid vector was linearized with BamHI, and chicken genomic DNA was partially digested with Sau3A I. The DNA fragments were ligated to the vector pWE15, to form concatemers. The DNA was packaged into phage lambda which was then used to infect *E. coli.*

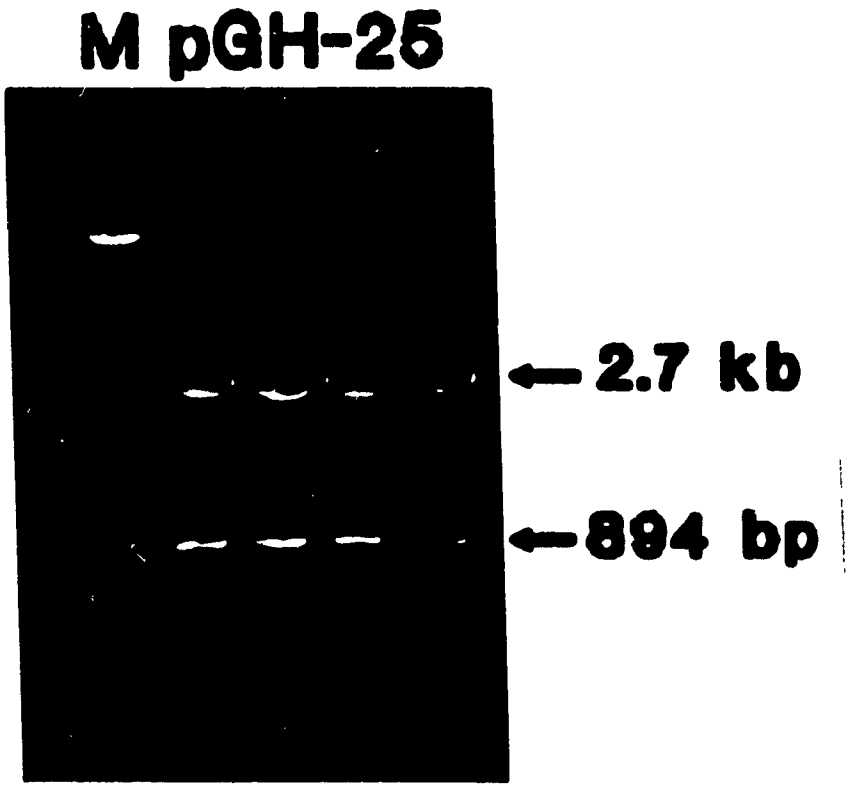

Fig. 3. Turkey GH cDNA gene (pGH-25) digested with EcoRI restriction enzyme, separated by electrophoresis in 1% low melting temperature agarose gel . The 2.7 kb fragment is vector pUC13, the 894 bp fragment is tGH cDNA gene which was used as a probe. M indicated lambda DNA double digested with EcoRI + HindIII.

1.3. Isolation of the chicken growth hormone gene from a cosmid library

1.3.1). Preparation of the membranes for colony screening

To screen the library a small aliquot of bacteria was removed from a stock of bacteria of the cosmid library stored at -70^{0}C without thawing out the stock. The aliquot was resuspended in LB medium, and titrated to find the concentration of the bacteria. The bacteria were diluted and plated on LB/Amp agar plates (1 l of LB medium contains 15 g bacto-agar, 100 x 15 mm plate diameter) to give about 1500 colonies. The plates were incubated overnight at 37^{0}C. The colonies were partially transferred to Hybond nylon membranes in replicate (Sombrook *et al.*, 1989). The first membrane was left in contact with the plate for one minute and the second one for two minutes. The membranes were marked with a needle for orientation. The bacteria on the membrane were lysed and the DNA denatured by treating the membrane in 0.5 N NaOH twice for 5 minutes each. The membranes were neutralized by soaking in 1 M Tris-HCl and 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5. The membranes were air-dried for 30 minutes, sandwiched between two sheets of 3 MM paper and baked at 80^{0}C for 2 hours.

1.3.2). Prehybridization and hybridization

Prehybridization, hybridization and washing were performed according to Kuhnlein *et al.* (1989). Briefly, the membranes were prehybridized at 42^{0}C for 2 to 4 hours before adding the probe. Following the addition of 2*10^6 cpm/ml of radiolabelled tGH, the hybridization was carried at 42^0 for 16 to 24 hours, in a

"rotissary" style incubator (Robbins Scientific). Prehybridization and hybridization buffer contained 24% deionized formamide, 4.8 x SSC, 20 mM Tris (pH 7.6), 0.5 x Denhardt's solution, 8% dextran sulphate, 0.1% SDS and 0.1 μg/ml denatured Herring sperm DNA.

The filters were washed at 42^{0}C for 15 minutes each with 100 ml of 2 x SSC/0.1% SDS; 0.5 x SSC/0.1% SDS; 0.1 x SSC/0.1% SDS. If the filter was still highly radioactive, it was washed at 52^{0}C with the last solution for another 30 minutes. The filters were air-dried and then subjected to autoradiography for 16-24 hours at -70^{0}C using Kodak X-ray film (XAR-5) and two DuPont Cronex intensifying screens. The films were developed for 2 minutes and fixed for 5 minutes.

The hybridisation signals were traced back to the corresponding colonies on the agar plate and the colonies were picked. In most cases it was not possible to pick single colonies. In these cases the bacteria from the corresponding area were suspended in LB broth and plated on LB/Amp plates at appropriate concentrations and the screening procedure was then repeated until a single positive colony could be isolated.

1.4. Subcloning of genomic fragments of the GH gene

1.4.1). Preparation of the chicken GH gene fragments and the vector

The purified GH-containing cosmid DNA was digested with PstI as described above and completeness of the digestion was tested by gel electrophoresis. The dephosphorylated vector pUC18 was prepared according to established protocols

(Sambrook *et al.*, 1989). In brief, 4 μg of pUC18 was digested to completion with 25 units of PstI restriction enzyme. The digested plasmid was heated at 65^0 for 10 minutes, chilled on ice and 0.17 μg of calf intestinal phosphatase (CIP) was added. The reaction was incubated at 37^0C for 40 minutes followed by heat inactivation of the enzyme by incubating at 85^0C for 15 minutes. The digested and dephosphorylated pUC18 was then gel-purified by standard procedures (Sambrook *et al.*, 1989).

1.4.2). Ligation of chicken GH gene fragment with pUC18

The digested cosmid DNA was ligated with the purified PstI-cut and dephosphorylated pUC18 vector. The ligation reaction contained 50 μg of pUC18 DNA, 100 μg of digested cosmid DNA, 0.5 mM ATP, 1x One-Phor-All buffer (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, and 50 mM potassium acetate) and 4 units T4 DNA ligase (Pharmacia) in a reaction volume of 10 μl. Ligation was carried out at 16 ^{0}C tor 16 hours. Two control reactions were included in the ligation. One contained pUC18 digested with PstI, the other contained pUC18 digested with PstI and dephosphorylated but no digested cosmid DNA.

1.4.3). Transformation

The ligated DNA was used to transform competent *E. coli* DH5α which had been prepared according to the method described by Sambrook *et al.* (1989). In brief, a 100 μl aliquot of competent cells which had been stored at -70 ^{0}C was thawed by warming the tube with the hand. It was then kept on ice for 10 minutes and 3 μl of

the ligation mixture was gently mixed with the competent cells by swirling. After a 30 minute incubation on ice, the cells were heat shocked by incubating at 42 ^{0}C for 90 seconds and chilled on ice for 2 minutes. An aliquot of 400 μl pre-warmed (37 ^{0}C) LB media was added to the cell suspension followed by incubation at 37 ^{0}C for 45 minutes with shaking. Aliquots of 50 μl and 200 μl of the cell suspension were then spread on LB/Amp plates and the plates were incubated at 37 ^{0}C overnight. A positive control with untreated pUC18 DNA was also included in the transformation reaction as were the controls for the ligation reaction. A negative control only included competent cells without DNA.

1.4.4). Screening of recombinant plasmid with GH cDNA

The screening procedure for identifying plasmids carrying GH gene fragments was the same as for screening the cosmid library (see III.1.3.).

1.5. Sequencing

The subclones of the chicken GH gene were sequenced by Sanger's dideoxy-mediated chain-termination method (Sambrook *et al.*, 1989) using the T_7 sequencing kit from Phamarcia. In brief, the template DNA (2 μg) was denatured with 0.4 M NaOH for 10 minutes and precipitated by adding 2 volumes of 100% cold ethanol and 0.1 volumes of 3 M sodium acetate. The pellet was washed with 70% ethanol, air dried and then redissolved in 10 μl of H_2O. Two μl of 0.8 μM sequence primers (Universal or Reverse primer), 2 μl of the annealing buffer containing 1.5 mM $MgCl_2$

and 1 mM DTT were added and the reaction incubated at 37^{0}C for 20 minutes. While the annealing reaction was incubating, four tubes were marked with "A", "C", "G" and "T" and 2.5 μl of each ddATP, ddCTP, ddGTP or ddTTP was pipeted into the corresponding tubes. After incubation at 37^{0}C, the annealing reaction was left at room temperature for 15 minutes. Then a mixture of dNTPs, ^{35}S-dATP and T_7 DNA polymerase was added and the incubation at room temperature continued for another 5 minutes to allow initiation of DNA synthesis from the primer. Immediately after the 5 minute incubation at room temperature, the reaction mixture was distributed into the four tubes containing the ddNTPs. The reaction was stopped by the addition of formamide. After denaturation, the fragments were separated by 8% polyacrylamide/urea (8 M) gel electrophoresis (Bio-Rad sequencing apparatus). The gel was run at 2200 V, at 50^{0}C for 2, 4 and 6 hours, respectively, fixed with 10% methanol/10% acetic acid and dried in a Bio-Rad gel drier. Autoradiography was carried out at room temperature for 16-24 hours.

1.6. Detection of RFLPs by using clones of the chicken genomic GH gene as probes

1.6.1). DNA samples of chicken

The fat line (FL) and lean line (LL) were derived by Leclercq (1988). These two lines were developed from a base population established from chickens of six different meat type strains of widely different origins. Selection for leanness and fatness was on the sire side and based on the weight of the abdominal fat pad. In each generation between 9 and 15 sires and 14 to 67 dams were used as breeders.

After selection for 6 generations, the two strains were imported to the poultry unit at Macdonald Campus in 1982 and were maintained without selection. Between 60 and 70 sires and the same number of dams were used to propagate each strain.

DNA was extracted from 100 μl of heparinized blood according to Jeffreys and Morton (1987). In brief, 100 μl of blood was diluted with 5 ml of 1 x SSC (0.5 M NaCl, 0.015 M sodium citrate, pH 7.0) and spun for 15 minutes at 7000 xg. The supernatant was discarded and the pellet was resuspended by vortexing in 2 ml of 0.2 M sodium acetate (pH 7.0). Then 100 μl 20% SDS was added to lyse the cells. The genomic DNA was extracted twice with one volume of phenol-chloroform-isoamyl alcohol (25:24:1 v/v) and once with chloroform-isoamyl alcohol, and precipitated with two volumes of 95% ethanol. The DNA was fished out with a pasteur pipette with a sealed end, washed twice with 70% ethanol, air-dried, and dissolved in TE buffer containing 5 mM Tris-HCl, 0.1 mM EDTA (pH 8.0). The concentration of DNA was measured in a spectrophotometer at a wavelength of 260 nm and 280 nm. and adjusted to a concentration of 250 μg/ml.

1.6.2). Digestion of genomic DNA by restriction enzymes

The genomic DNA (5 μg in 20 μl) was digested with 25 units of restriction enzyme in the presence of 2.5 μl 10 x One-Phor-All buffer (see above). After an overnight incubation at 37 ^{0}C the sample was heated at 85^{0}C for 10 minutes and chilled on ice.

1.6.3). Gel electrophoresis

To each sample, 5 μl of Ficoll loading buffer (15% Ficoll type 400 (W/V), 0.25% xylene cyanol (w/v), 0.25% bromophenol blue (w/v)) was added. The DNA fragments were separated by electrophoresis in a 1% agarose gel. The gel was prepared in 1 x TPE buffer (80 mM Tris-phosphate, 8 mM EDTA, pH 8.0; Sambrook *et al.*, 1989) by melting the agarose in a microwave oven for 5-6 minutes. The agarose was cooled to 50 ^{0}C and poured into a gel tray (size 20 cm x 15 cm) containing a 15 or 20 well comb. After the agarose gel solidified, the tray with the gel was placed into the electrophoresis subcell (Bio-Rad). Buffer (1 x TPE) was added until the gel was submersed by about 5 mm. The DNA samples were loaded into the wells and the gel was run at 29 V for 18 hours. After completion of the electrophores' , the gel was stained with 0.5 μg/ml ethidium bromide for 15 minutes and the quality of the digestion checked under UV light.

1.6.4). Southern Blotting

The DNA was transferred from the agarose gel onto a Zeta probe membrane (Bio-Rad) by alkali blotting (Southern, 1975). In brief, the gel was trimmed to 14 x 17 cm and treated with 200 ml of 0.25 M HCl for 25 minutes to depurinate the DNA. Capillary blotting was carried out in a "transfer tower" built in a tray as follows: 4 sheets of 3 MM paper as wicks, gel (upside down), zeta-probe membrane (pre-treated with hot 0.1% SDS), 4 sheets of 3 MM paper (size of the gel), 10 cm stack of paper towels, glass plate, and a weight of about 250 g. A solution of 0.4 M NaOH

was placed into the tray. Air bubbles were removed by rolling gently with a pipet over the surface of each layer. After 20 hours at room temperature, the membrane was removed and rinsed with 2 x SSC containing 0.1% SDS.

1.6.5). Prehybridization and hybridization

The prehybridization and hybridization procedure was the same as described in section III.1.3.2), but the buffers were different. The prehybridization buffer contained 5 μg/ml skim milk powder (Carnation), 4 x SSPE (0.72 M NaCl, 40 mM sodium phosphate pH 7.4, 4 mM EDTA), 50% deionized formamide, 1% SDS and 0.5 μg/ml herring sperm DNA. The hybridization buffer was the same as the prehybridization buffer, except that it contained 10% dextran sulphate, $2*10^6$ cpm/ml heat denatured radioactive probe and no herring sperm DNA.

1.7. PCR amplification of target sequences from GH gene loci

A PCR reaction was designed to amplify a 629 base pair region from the chicken GH gene which contained the PM3 polymorphic site. Two primers located in the 5' flanking region and the first intron of GH gene, respectively, were designed based on the genomic sequence of Tanaka *et al.* (1992) (Fig. 8). The sequence of the forward primer (PM3F) was 5'-GTTGCAAATAACAGCAGAAT-3' and the reverse primer (PM3R) 5'-CCTCGACATCCAGCTCACAT-3'. A second pair of primers was designed to amplify a 690 bp fragment which contained the PM1 polymorphic site. PM1F (5'-TCCTTTCTTGGATGTGTTCA-3') and PM1R (5'-

GTGATGCTGTCTTTGCTCCA-3') were both located in the fourth intron.

In the PCR reaction, 200 ng genomic DNA was used in 100 μl of a reaction mixture containing 1.25 μM of each primer, 10 μl of 10 x reaction buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl), 5 mM of each dNTP, 5% formamide, 2.5 units of *Tth* DNA polymerase (Pharmacia). The reaction solution was overlaid with a drop of mineral oil to prevent evaporation. The reaction was carried out in a thermal cycler (Perkin Elmer Cetus Corp, New Jersey, USA). The reaction mixture was first heated at 95^0C for 3 minutes, *Tth* DNA polymerase was added followed by 35 cycles. Each cycle consisted of 30 seconds at 94^0C (denaturation), 2 minutes at 56^0C (annealing) and 2 minutes at 72^0C (extension).

1.8. Analysis of the PCR products amplified from GH gene

Aliquots of 10 μl of the PCR products were digested with 7 units of MspI restriction enzyme without adding additional buffer at 37^0C for 2 hours. The samples were heated at 65^0C for 10 minutes and chilled on ice. Two μl of 6 x loading buffer containing 30% glycerol and 0.25% bromophenol blue were added. The fragments were separated by electrophoresis on a 7% polyacrylamide gel (PAG) (Sambrook *et al.*, 1989) at 140 volts for 1.5 hour.

Following electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml), and visualized under UV light and photographed with a polaroid MP-4 camera and type 400 film at an aperture of 5.4 and an exposure time of one second.

2. Results and discussion

2.1. Isolation of the chicken GH gene

2.1.1). Identification of GH clones in a genomic cosmid library

Screening of about 50,000 colonies yielded four colonies which hybridized to the turkey GH cDNA probe. Since it was not possible to pick individual colonies, bacteria from the potential-positive areas were streaked out on a plate. Single colonies were picked, spotted on a plate, incubated and screened again. Fig. 4 shows an example of such a second screen. The cosmid DNA was purified from colonies which were positive in the second screen. It was digested with different enzymes, blotted onto nylon membrane and hybridized again with tGH cDNA. Of four cosmids, three had exactly the same structure and one was a false positive.

2.1.2). Subcloning of the PstI fragments of the GH gene

Fig. 5 showed the subcloning strategy. First, I tried to isolate and purify single bands and subclone them into pUC18. However, several attempts failed, presumably because the recovery of DNA was too low or the DNA was not purified enough. The PstI-digested cosmid DNA was therefore directly ligated with the dephosphorylated and linearized pUC18 plasmid to establish a sub-genomic library (Fig. 5). The ligation mixture was used to transform competent *E.coli* DH5α cells. After several rounds of screening with the turkey GH cDNA probe, several positive subclones were identified which had insert size of about 1600 bp or 1500 bp. These positive subclones had inserts with the same size as PII and PI. Another subclone was isolated from the

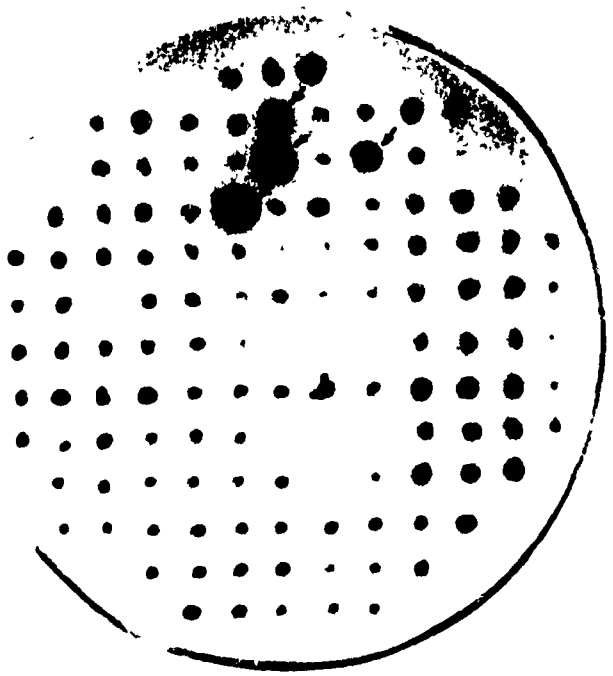

Fig. 4. Second screen showing positive clones. The arrows indicate positive colonies containing GH gene recombinant cosmids.

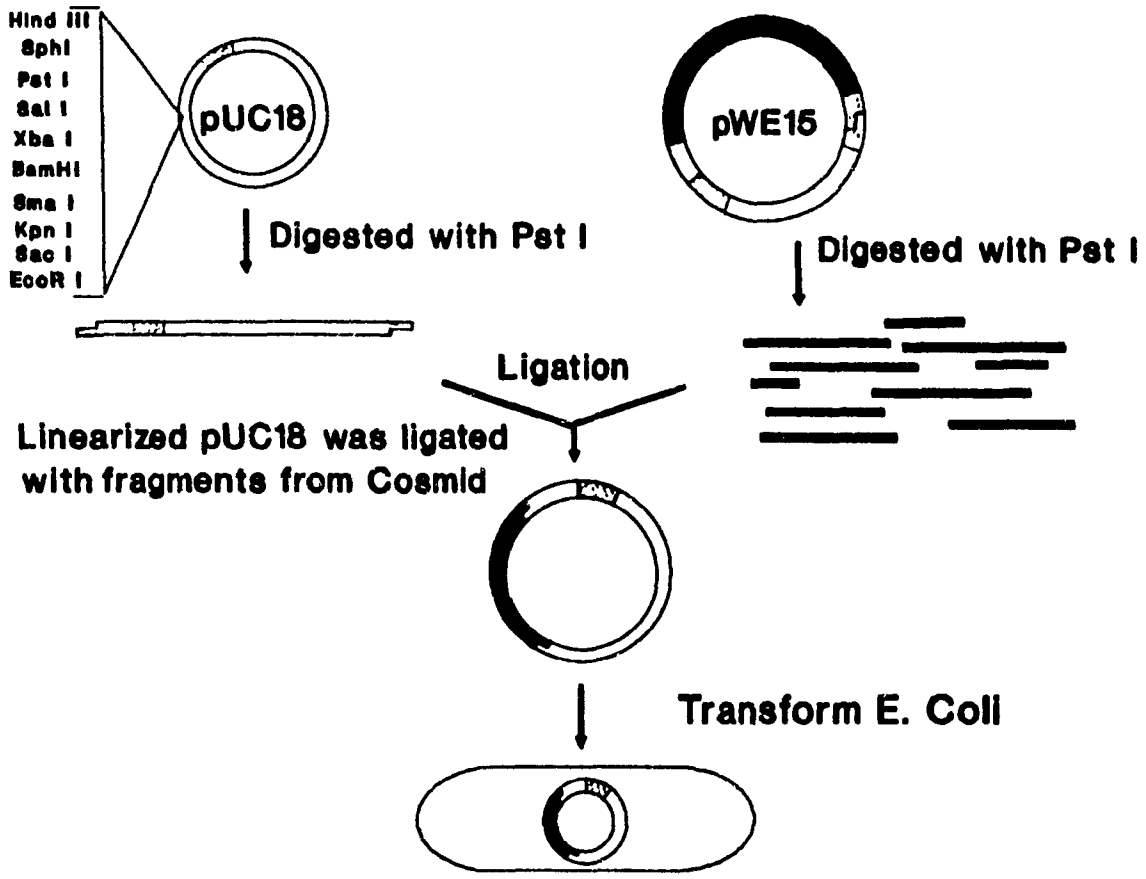

Fig. 5. Subcloning strategy of GH gene. The GH cosmid DNA was digested with PstI and ligated to the PstI-digested and dephosphorylated plasmid vector pUC18 to form a sub-genomic library. This sub-library was screened with tGH cDNA probe using the same procedure as in screening for positive cosmid clones.

sub-genomic library by using the cosmid fragment PIII as a probe (Fig. 10). The clones containing PI, PII and PIII insert are referred to as pUCGH2, pUCGH4 and pUCGH19.

2.1.3). Restriction map of the cloned genomic GH gene

A restriction map of the cloned GH gene together with its flanking regions was obtained by analyzing partial PstI digests, single-, double- and triple-digests of the cosmid with PstI, BamHI, SacI and MspI. The probes used in these analyses were the PstI subcloned GH gene fragments PI, PII, PIII and tGH cDNA. The autoradiographs from which the maps were derived are show in Figs. 6, 7, 8 and 10. All the fragments whose arrangement is discussed below are indicated in Figs. 9 and 11.

Hybridization with clone PII is shown in Fig. 6. Digestion with PstI, SacI or BamHI produced a single strongly hybridizing fragments of molecular weight (MW) 1.65 kb (P2), 3.8 kb (B1) and 4.6 kb (S1), respectively. Both PstI/BamHI and PstI/SacI double digestion also produced P2, hence the fragment P2 is entirely contained in fragments B1 and S1. BamHI and SacI double digestion yielded BS1 (3.6 kb), the fragment B1 has a SacI site within 200 bp of either end. As expected from this arrangement, triple digestion with PstI, BamHI and SacI yielded P2.

MspI digestion yielded two fragments M1 (7.8 kb) and M2 (1.8 kb). Double digestion with MspI and BamHI yielded two new fragments, BM1 (3.5 kb) and BM2 (0.66 kb), indicating that both MspI fragments have a BamHI site. Since BM1 is

larger than M2, it must have originated from M1 and hence BM2 from M2.

Further, PstI/MspI digestion yielded two new fragments PM1 (1.3 kb) and PM2 (0.46 kb). Since PM1 is larger than PM2, it must be on the same side BM1. The same argument allows to orient SM1 and SM2 in respect to BM1 and BM2. As expected, triple digestion with PstI, BamHI and MspI yielded PM1 and PM2.

Hybridization with PIII is shown in Fig. 7. Single digests yielded fragments P3 (1.05 kb), B1, S1 and M1. In double digests, P3, BS1, BM1 and SM1 are observed. This pattern is expected if P3 is located to the left of P2 between the first SacI site and the first PstI site. Partial digests with PstI yielded a fragment of 2.65 kb which is the sum of P2 and P3. Hence the two fragments are presumably adjacent to each other.

Hybridization with PI is shown in Fig. 8. PstI and BamHI digestion yielded single hybridizing fragments of 1.5 kb (P1) and 6.4 kb (B2), respectively. The double digest with PstI/BamHI yielded a fragment of the size of P1, indicating that P1 is nested within the fragment B2. SacI digestion yielded the fragment S1 (4.6 kb) and two new fragments S2 (2.4 kb) and S3 (0.45 kb). Since double digestion PstI/SacI did not affect S3, it must be nested within P1. As expected from this arrangement, two additional fragments PS1 (0.9 kb) and PS2 (0.34 kb) are observed. Based on signal strength, PS1 must originate from S2 and PS2 from S1. Thus, there are two SacI sites in the fragment P1 oriented as shown in Fig. 9.

MspI digestion yielded M2 (1.8 kb) and two new fragments, M3 (0.81 kb) and M4 (0.32 kb). Double digestion with MspI and PstI did not affect M4 but yielded two new

fragments PM3 (0.8 kb) and PM4 (0.5 kb). Based on signal strength PM3 originates from M3 and PM4 from M2. Thus there are also two MspI sites in P1 orientated as shown in Fig. 9.

The BamHI/SacI double digestion yielded S2 and S3 and a new fragment BS2 (1.05 kb). It indicates that S2 and S3 are within B1 and that S1 has a BamHI site.

With this information a map can be drawn and all the fragments observed in the digestions with the other restriction enzymes can be explained and are indicated in Fig. 9.

Hybridization with tGH cDNA is shown in Fig. 10. Single PstI digestion yielded fragments P1, P2 and a new fragment of 1 kb (P4), this new fragment may be located between fragments P1 and P2. The P3 fragment did not hybridize with tGH cDNA. BamHI digestion yielded B1, B2 and a cross-hybridisation fragment (2.5 kb). SacI digestion yielded fragments S1 and S3. MspI digestion yielded fragments M1, M2 and M4.

PstI/BamHI double digestion yielded fragments P1 and P2 PstI/SacI double digestion yielded fragments P2, P4 and S3. PstI digestion with MspI yielded fragments PM1, P4, PM4, PM2 and M4. This result indicates that in fragment P2 has a MspI restriction site and in fragment P1 has two MspI sites. BamHI/SacI double digestion yielded fragments BS1 and S3. BamHI/MspI double digestion yielded fragments BM1, BM2, BM3 and M4. SacI/MspI double digestion yielded SM1, SM2, SM4 and M4. PstI/BamHI/SacI triple digestion yielded fragments P2, S3 and PS2. PstI/SacI/MspI triple digestion yielded PM1, P4, PM2 and SM4. The fragments P3, S2, M3, PS1,

Fig. 6. Southern blot of the GH cosmid clone digested with PstI (P), BamHI (B), SacI (S) and MspI (M) restriction enzymes, or partially digested with PstI for 15 and 30 minutes. The filter was hybridized with the subcloned chicken GH gene fragment PII.

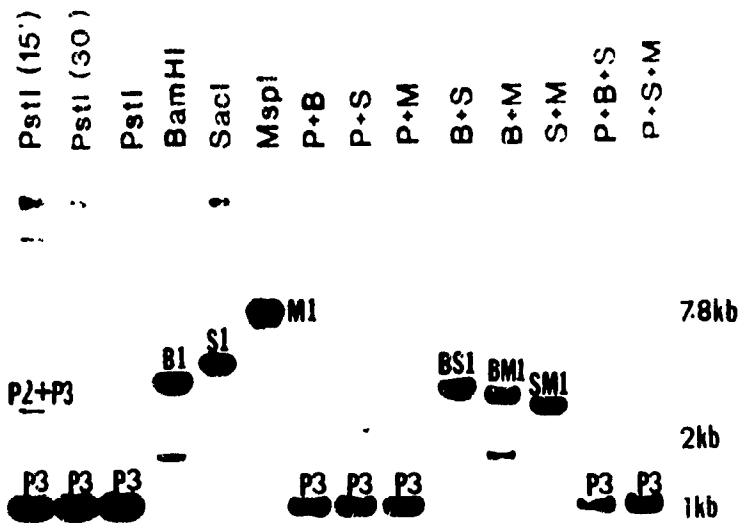

Fig. 7. Southern blot of the GH cosmid clone digested with PstI (P), BamHI (B), SacI (S) and MspI (M) restriction enzymes, or partially digested with PstI for 15 and 30 minutes. The filter was hybridized with the subcloned chicken GH gene fragment PIII.

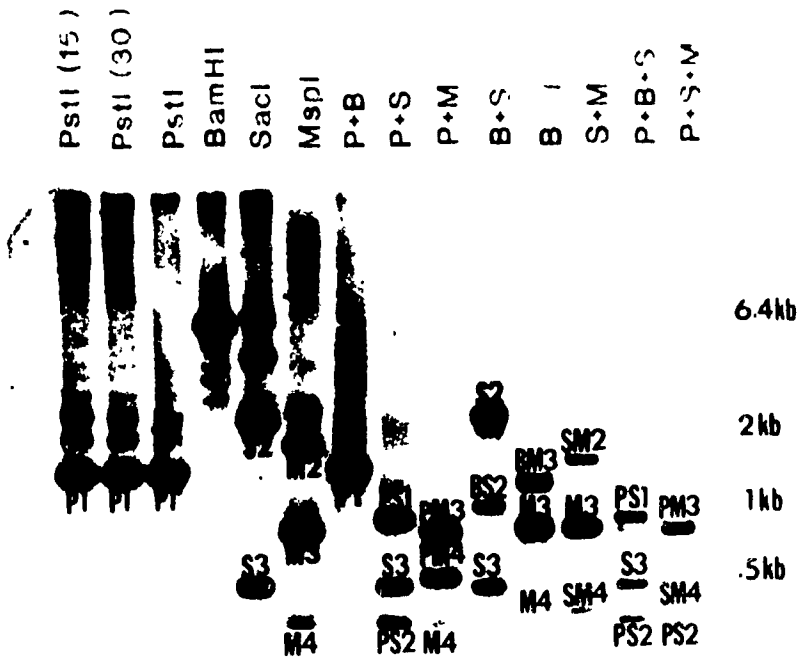

Fig. 8. Southern blot of the GH cosmid clone digested with PstI (P), BamHI (B), SacI (S) and MspI (M) restriction enzymes, or partially digested with PstI for 15 and 30 minutes. The filter was hybridized with the subcloned chicken GH gene fragment PI.

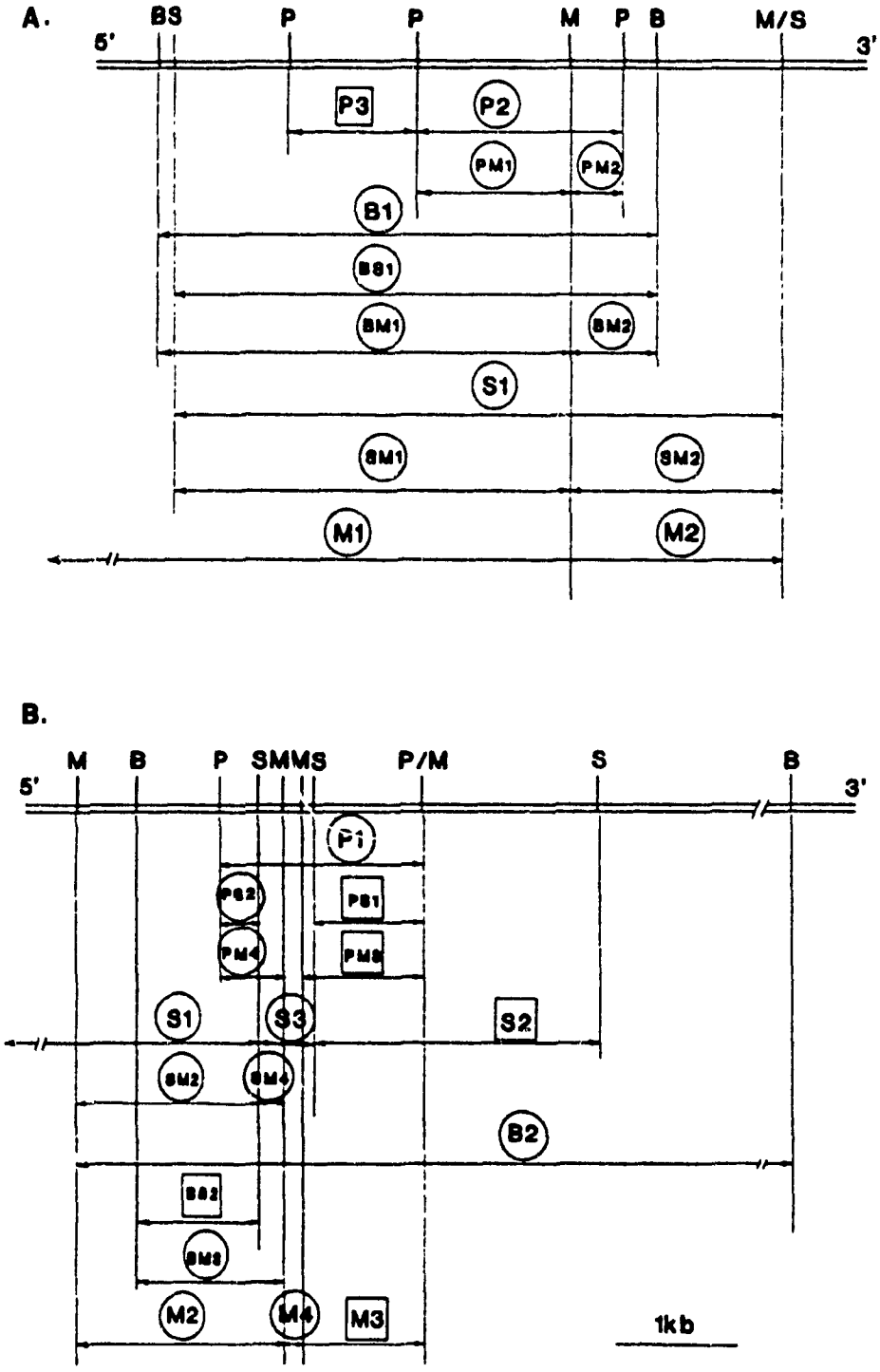

Fig. 9. Restriction map of GH gene. The restriction sites indicated on the map are PstI (P), BamHI (B), SacI (S) and MspI (M). Map A was established by using the subcloned GH gene fragments PII, PIII and is located in the 5' side of the GH gene. Map B was established by using subcloned GH gene fragment PI and is located in the 3' side of the GH gene. The fragments marked with square boxes are the fragments which only hybridized with subcloned PstI fragments, while the fragments marked with circles are the fragments which hybridized with both subcloned PstI fragments and the turkey GH cDNA probe.

Fig. 10. Southern blot of the GH cosmid clone digested with PstI (P), BamHI (B), SacI (S) and MspI (M) restriction enzymes, or partially digested with PstI for 15 and 30 minutes. The filter was hybridized with turkey GH cDNA.

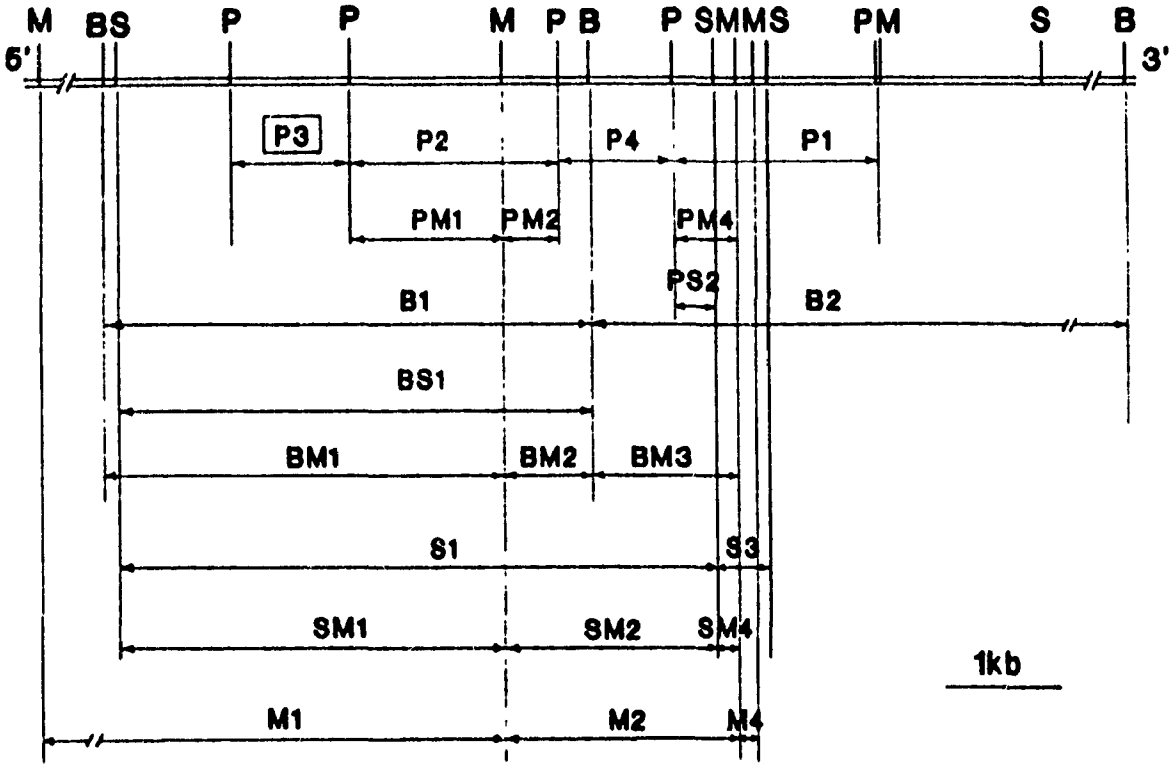

Fig. 11. Composite restriction map of the cloned GH gene. The restriction sites indicated on the map are PstI (P), BamHI (B), SacI (S) and MspI (M). P1 (1.5 kb), P2 (1.6 kb), and P3 (1.0 kb) indicate the PstI digestion fragments which were subcloned PI, PII and PIII, respectively. The P3 fragment did not hybridize with the turkey GH cDNA probe, indicating that it did not contain cDNA sequence.

PM3 and BS2 (Fig. 9) did not hybridize with tGH cDNA probe, indicating that these fragments are located in introns or flanking regions.

From a comparison of the fragments above with Figs. 6, 7, 8 and 9 a complete restriction map of chicken GH gene with its flanking regions was obtained (Fig. 11). The restriction map was in agreement with the map of Fotouhi *et al.* (1993) which had been established from the hybridization pattern of genomic DNA with tGH cDNA.

2.1.4). Analysis of the chicken GH gene sequence

The positive subclones pUCGH4, pUCGH2 and pUCGH19 were sequenced by using the M13 universal and the reverse sequencing primers. The number of base pairs sequenced were 1070 for pUCGH4 (700 bp from the universal primer and 370 bp from the reverse primer, Fig. 13); 640 for pUCGH2 (420 bp from universal primer and 220 bp from reverse primer, Fig. 14); 540 for pUCGH19 (290 bp from the universal primer and 250 bp from the reverse primer, Fig. 15).

While sequencing of the subclones was in progress, the chicken genomic GH sequence was published by Tanaka *et al.* (1992). Sequence comparison revealed that pUCGH4 extended from a PstI site in the first intron to a PstI site in the third intron. Based on optimal homology, the sequence starting from the universal primer side (Fig. 13: PUCGH4U.SEQ) corresponded to the sequence of Tanaka *et al.* (1992) starting from position 1050 in the 3'-direction (Fig. 12). The 700 base pairs sequenced revealed 95.9% homology. The sequence from the reverse primer (Fig. 13:

PUCGH4R.SEQ) corresponded to the sequence starting from position 1756 in the 5'-direction (Fig. 12). Only the first 258 bases of the 370 base pair sequence are homologous to the sequence of Tanaka *et al.* (1992).

The insert of pUCGH2 was located in the fourth intron, starting at position 2758 and extended past the published sequence in the 3'-direction (Fig. 12). The 420 base pairs sequenced from the universal primer (Fig. 14: PUCGH2U.SEQ) had 97.9% homology with the sequence of Tanaka *et al.* (1992). The 250 base pairs sequenced from the reverse primer (Fig. 14: PUCGH2R.SEQ) were 3' to the reported sequence. With pUCGH19 no homology with the sequence of Tanaka *et al.* (1992) was found.

Since the primary goal of the sequencing work was to develop primers for the purpose of developing PCR assays for RFLPs in the GH gene which are associated with traits, the sequence work was stopped without attempting to solve discrepancies as soon as the work of Tanaka *et al.* (1992) was published. It has to be noted, however, that sequencing of a PCR product (see below), indicated the presence of a 200 bp sequence in the 5'-proximal part of the GH gene which is not present in the sequence of Tanaka *et al.* (1992). It indicates that there may be extensive differences in the genomic GH gene between different strains of chickens and pursuit of further sequence work may be warranted.

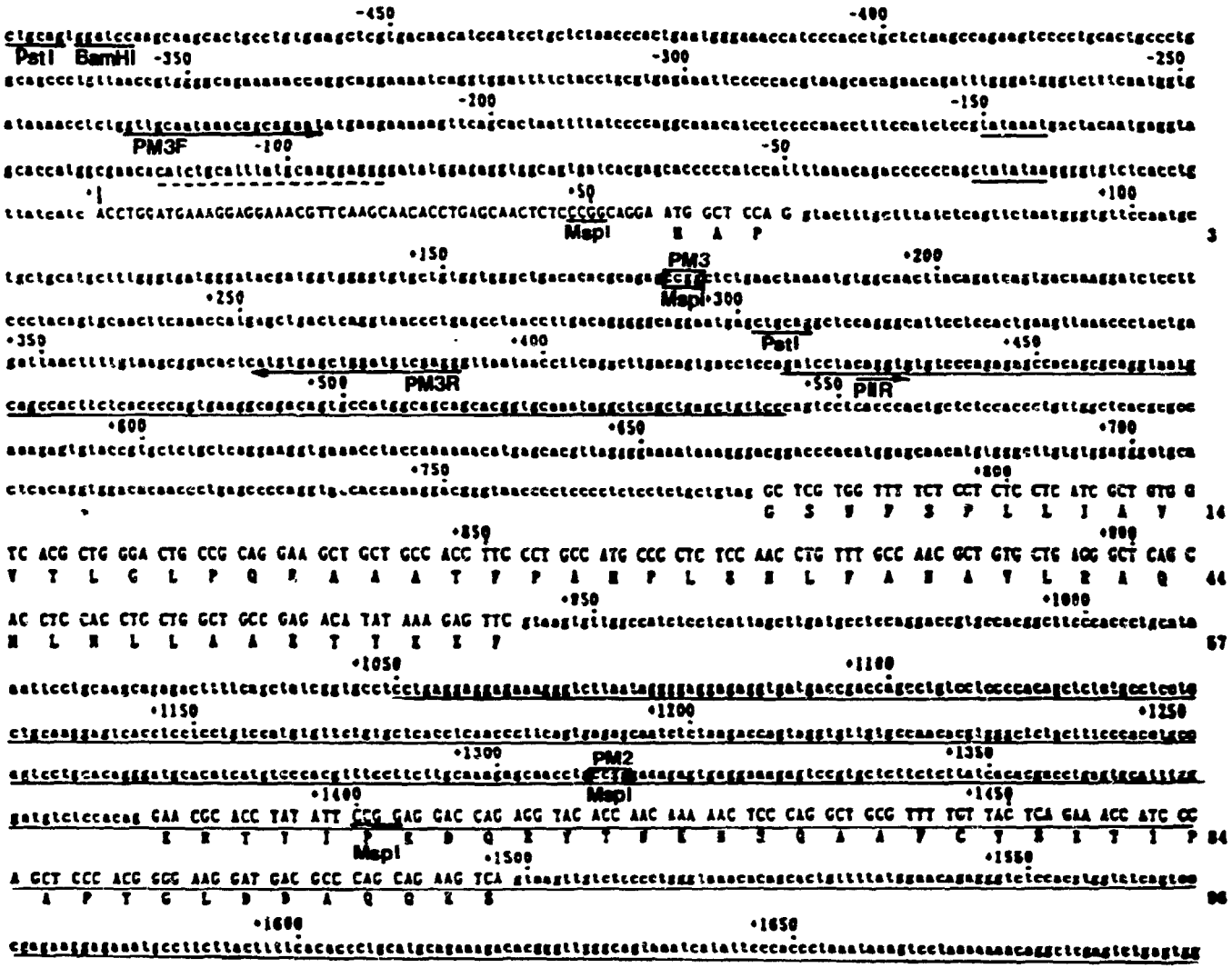

Fig. 12a. Figure legend is the same as Fig. 12b.

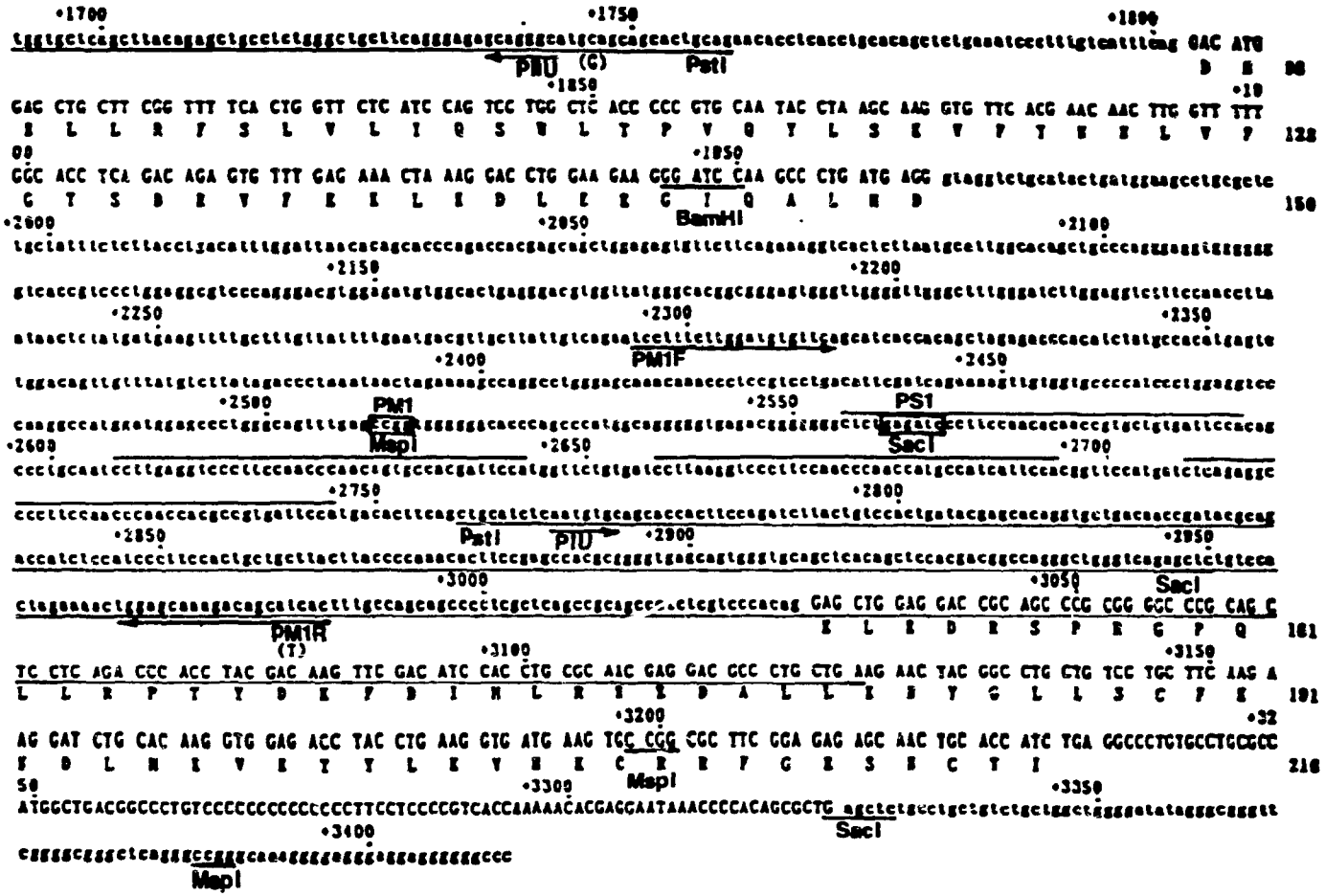

Fig. 12b. The chicken GH gene sequence (from Takana *et al.*, 1992) showing introns (small letters) and exons (capital letters). The areas of homology with the PstI clones are underlined (PII from position +426 to +1757, PI from position +2758 to the 3' end). Polymorphic sites are marked with boxes (PM1, PM2, PM3 and PS1) and PCR primer locations are underlined with arrows.

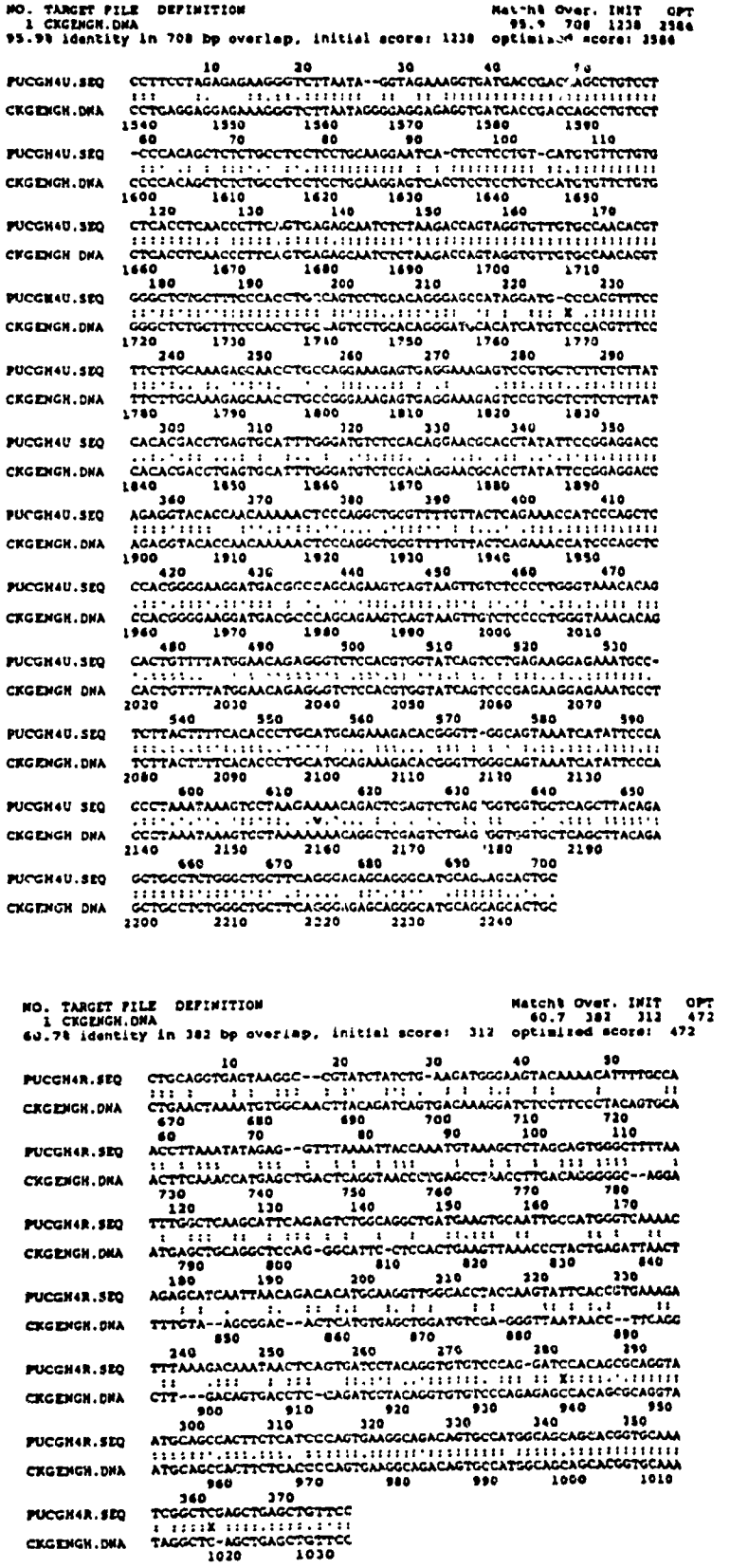

Fig. 13. Comparison of the sequence of pUCGH4 (PII) with the genomic sequence of Tanaka *et al* (1992). Position 1540 corresponds to position +1050 and 670 to position +179 in Fig. 12.

```
FILE NAME  : PUCGH2U.SEQ
SEQUENCE   :   427 BP;     96 A;     151 C;     105 G;     75 T;
RANGE      :     1 -   427          MODE     : NORMAL

CUTOFF     :    16                  KTUP     : 4

TARGET     : sequences on data disk

*** HOMOLOGY REGIONS REFERENCE ***

NO. TARGET FILE  DEFINITION                                  Match% Over. INIT   OPT
  1 CKGENGH.DNA                                               97.9  427  1654  1654
97.9% identity in 427 bp overlap, initial score: 1654  optimized score: 1654

                10        20        30        40        50        60
PUCGH2U.SEQ     CTGCAGCTCCATGTGCAGCACCACTTCCAGATCTTACTGTCCACTGATACGAGCGCAGGT
                X:::: ::: :::::::::::::::::::::::::::::::::::::::::: :::::
CKGENGH.DNA     CTGCATCTCAATGTGCAGCACCACTTCCAGATCTTACTGTCCACTGATACGAGCACAGGT
                 3250      3260      3270      3280      3290      3300
                70        80        90        100       110       120
PUCGH2U.SEQ     GCTGACAACCGATACGCAGACCATCTCCATCCCTTCCACTGCTGCTTACTTACCCCAAAC
                ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CKGENGH.DNA     GCTGACAACCGATACGCAGACCATCTCCATCCCTTCCACTGCTGCTTACTTACCCCAAAC
                 3310      3320      3330      3340      3350      3360
                130       140       150       160       170       180
PUCGH2U.SEQ     TCTTCCGAGCCACGCTGGGTGAGCAGTGGGTGCAGCTCACAGCTCCACGACGTCCAGGGC
                ::::::::::::::: :::::::::::::::::::::::::::::::::: :::::::
CKGENGH.DNA     ACTTCCGAGCCACGCGGGGTGAGCAGTGGGTGCAGCTCACAGCTCCACGACGGCCAGGGC
                 3370      3380      3390      3400      3410      3420
                190       200       210       220       230       240
PUCGH2U.SEQ     TGGGTCAGAGCTCTGTCCACTAGAAAACTGGAGCAAAGACAGCATCAGTTTGCCAGCAGC
                :::::::::::::::::::::::::::::::::::::::::: ::::::::::::
CKGENGH.DNA     TGGGTCAGAGCTCTGTCCACTAGAAAACTGGAGCAAAGACAGCATCACTTTGCCAGCAGC
                 3430      3440      3450      3460      3470      3480
                250       260       270       280       290       300
PUCGH2U.SEQ     CCCTCCCTCAGCCGCAGCCCTCTCGTCCCACAGGAGCTGGAGGACCGCAGCCCGCGGGGC
                ::::: ::::::::::::::::::::::::::::::::::::::::::::::::::::
CKGENGH.DNA     CCCTCGCTCAGCCGCAGCCCTCTCGTCCCACAGGAGCTGGAGGACCGCAGCCCGCGGGGC
                 3490      3500      3510      3520      3530      3540
                310       320       330       340       350       360
PUCGH2U.SEQ     CCGCAGCTCCTCAGACCCACCTACGACAAGTTCGACATCCACCTGCGCAAGGAGGACGCC
                ::::::::::::::::::::::::::::::::::::::::::::::::::: ::::::::
CKGENGH.DNA     CCGCAGCTCCTCAGACCCACCTACGACAAGTTCGACATCCACCTGCGCAACGAGGACGCC
                 3550      3560      3570      3580      3590      3600
                370       380       390       400       410       420
PUCGH2U.SEQ     CTGCTGAAGAACTACGGCCTGCTGTCCTGCTTCAAGAAGGATCTGCACAAGGTGGAGACC
                ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
CKGENGH.DNA     CTGCTGAAGAACTACGGCCTGCTGTCCTGCTTCAAGAAGGATCTGCACAAGGTGGAGACC
                 3610      3620      3630      3640      3650      3660

PUCGH2U.SEQ     TACCTGA
                :::::::X
CKGENGH.DNA     TACCTGA
                 3670


FILE NAME : PUCGH2R.SEQ

SEQUENCE  :   224BP;     42 A;     102 C;     36 G;     43 T.

  *** SEQUENCE LIST ***                    (SINGLE)

          10        20        30        40        50        60
5' CTGCAGGTCC CACCCTCACC CTTCAGCACC AGGGGTCTGG GCCCCCACTC CTCAACCCCC
          70        80        90        100       110       120
   AAGAGGGTTC TGCTCCCCAA ACACAGCTCT AGCCTGCTCA TCCCAGACCC TTCCCCAGGC
         130       140       150       160       170       180
   TCCTGATCCC ATCCTCAGGT CTGATGGTCA TCCCATTNCA GTCCTCATCC CCAGGCCCAG
         190       200       210       220
   TTCCCCAGTC TCAGACCCAG GTGCCAACCC CACCCTATTC CCAA 3'
```

Fig. 14. Comparison of the sequence of the sub-clone pUCGH2 (PI) with the sequence reported by Tanaka *et al* (1992). Position 3250 corresponds to position +2758 in Fig. 12. The sequence starting from the reverse primer (PUCGH2R) was 3' to the sequence determined by Tanaka *et al.* (1992).

```
FILE NAME : PUCGH19U.SEQ

SEQUENCE  :   295BP;     71 A;     64 C;     87 G;     73 T.

  *** SEQUENCE LIST ***                (SINGLE)

            10          20          30          40          50          60
5' CTGCAGAATG ATGTAACCCT TGGCAGAGCA GTCTGGTAAG TTAGGTATTG TATTTGTTGG
            70          80          90         100         110         120
   CTCAGTTCAG GAAATGCGTA AGTTAAATCT CCATTGTGTC CTCTACAGTT CTGTCAGGGG
           130         140         150         160         170         180
   GCAGCATGGG CTGTAGGACT CCCTTGGGGT TGTGGAGCTG GAGCTGAGGG AGACCTAGAC
           190         200         210         220         230         240
   CTGATCATAC TCTCACCCAC AGGGCACAGT GTCTTGGGGC TAGTGATGGG ATCATTCATT
           250         260         270         280         290
   AGCCCACAAC ATGGCAAGTG AATGAACCAG GGCCAGGGAA AAACCCAGGG TCTCC 3'

        FILE NAME : PUCGH19R.SEQ

        SEQUENCE : 250BP;   70 A;   42 C;   44 G;   94 T.

          *** SEQUENCE LIST ***              (SINGLE)

                    10          20          30          40          50
        5' CTGCATATAA GCTGTGCAAA TGCGCACTTG GAAATGCACA ACTAATTTGT
                    60          70          80          90         100
           GGAGCTGAGA TCATTTAAAA TGTGAACTTG GTCAGTACCT GAGATCTTTT
                   110         120         130         140         150
           ATATNTTCTA AGATAGTTTC AGAGCCTGTA TATACTTAAC ATTTCTCTCT
                   160         170         180         190         200
           GAAAGTTTTT TTGTTAACAT TCCATTCATT AAAATTGTGA GTCTGATGAG
                   210         220         230         240         250
           TGTCTCATAT ACTAAGTCGG TAGATTTCCA CATTCAGCTC CCTTTTTGTG
```

Fig. 15. The sequence of the sub-clone pUCGH19. The 295 bp read from the universal primer and the 250 bp from reverse primer had no homology with the sequence of Tanaka et al. (1992). The corresponding fragment in the restriction map (Fig. 11) is PIII.

2.2. Analysis of chicken genomic DNA by hybridization with chicken genomic GH gene fragments

Fotouhi *et al.* (1993) searched for growth hormone DNA polymorphism using the turkey GH cDNA gene as a probe. Four polymorphic sites were identified, one at the SacI restriction site (PS1), three at the MspI restriction sites (PM1, PM2, and PM3), located in the first, second and fourth intron, respectively. The individual genotypes were first derived by comparing band presence or absence (homozygote vs heterozygote) and band associations, and then confirmed by restriction mapping of some selected individuals. Table 1 shows GH genotypes of four individuals from two strains developed by Leclereq (1988) at four polymorphic sites (Fotouhi *et al.*, 1993). Chickens #8 and #10 were from the fat line and #21 and #25 from the lean line. At the PS1 restriction site, all individuals are homozygous for the absence of the restriction site (+/-)(-/-) except #8, which was heterozygous. At the PM1 site, the individuals were -/- homozygotes except #21, which was a heterozygote (+/-). At the PM2 site, chickens #8 and #10 are +/+ homozygotes, whereas, chickens #21 and #25 are heterozygotes. At the PM3 site, chickens #8 and #25 were -/- homozygotes, and #10 was a +/+ homozygote and #21 was a heterozygote.

Fotouhi *et al.*, (1993) found that the frequency of some polymorphisms of the GH gene were correlated to disease resistance, egg production and age at sexual maturity of chickens. PS1 and PM3 were found to be associated with resistance to Avian Leukosis virus (ALV) and Marek's disease (MD). Significant correlations were also found between the age at sexual maturity and frequency of homozygotes for one of

the PM3 polymorphisms (Kuhnlein *et al.*, 1993). The polymorphism at PM1 and PM2 were fixed in the fat line but segregated in the lean line (Fotouhi *et al.*, 1993). All these polymorphic sites can potentially be used as genetic markers in poultry breeding programs.

To investigate whether the cloned chicken GH gene fragments can be used for detecting these RFLPs in the GH gene, genomic DNA from two chickens of the fat line (#8 and #10) and the lean line (#21 and #25) of Leclercq (1988) were analyzed for RFLPs using the fragments PI, PII and PIII as probes. Each sample was digested with restriction enzymes PstI, BamHI, SacI and MspI, or double digested with pair-wise combinations of these enzymes, respectively, and analyzed using the standard Southern blotting procedure. The restriction map based on the analysis of the cloned GH gene (Fig. 11), the sequence of Tanaka *et al.* (1992) and the analysis of Fotouhi *et al.* (1993) is shown in Fig. 16. It indicates the fragments expected to be observed when hybridyzing with the cloned chicken GH gene fragments.

Fig. 17 and 18 show the result obtained with PI as a probe, which represents 1.5 kb downstream of intron IV of the GH gene (Fig. 10). Although some major bands which may represent hybridization to GH gene fragments were observed, the blot revealed multiple minor bands whose sizes varied with the restriction enzyme used for digestion. It indicates the presence of repetitive sequences in PI which may hybridize to other regions of the genome. These repetitive sequences may be in the unsequenced part of the PI fragment. Hence PI can not be used for RFLP analysis.

Fig. 19 and 20 show the results from hybridization with PII. With the exception

of a set of weak bands which we attribute to cross-hybridization, all observed bands and the corresponding molecular weights agree with the map shown in Fig. 16.

PII appears to be an ideal probe to analyze the previously described GH RFLPs. In particular, SacI digestion of the chicken #8 which is PS1+/PS1- shows two bands, S2 and S1, while the other three chickens which are homozygous for PS1- only show band S1.

Genomic DNA digested with MspI produces more complex patterns. PII is expected to reveal RFLPs at PM2 and PM3. As expected, chicken #8 (PM1-/PM1-; PM2+/PM2+; PM3-/PM3-) produces M2 and M1, chicken #10 (PM1-/PM1-; PM2+/PM2+; PM3+/PM3+) produces M3 and M1 and chicken #25 (PM1-/PM1-; PM2+/PM2-; PM3-/PM3-) produces M1, M2 and M4. Chicken #21 is heterozygous for all three MspI polymorphisms. It produces bands M1, M2, M5 and M6. Since M2 and M6 rather then M3 and M4 are observed, the two gametes must be (PM2+, PM3-) and (PM2-, PM3+) respectively and not (PM3+, PM2+) and (PM2-, PM2-).

Table 1. The genotypes of chickens used in Southern blotting analysis[1]

Line[2]		Genotype			
		PS1	PM1	PM2	PM3
Fat line	#8	+/-	-/-	+/+	-/-
	#10	-/-	-/-	+/+	+/+
Lean line	#21	-/-	+/-	+/-	+/-
	#25	-/-	-/-	+/-	-/-

[1] The genotypes were determined by Fotouhi *et al.* (1993). PS1 designates a SacI site and PM1, PM2 and PM3 designate MspI restriction sites where polymorphisms were observed. '+' indicates the presence of a restriction site and '-' the absence of a restriction site.

[2] The fat line and lean line are two lines developed by Leclercq (1988) from a common genetic base population and divergently selected for fatness.

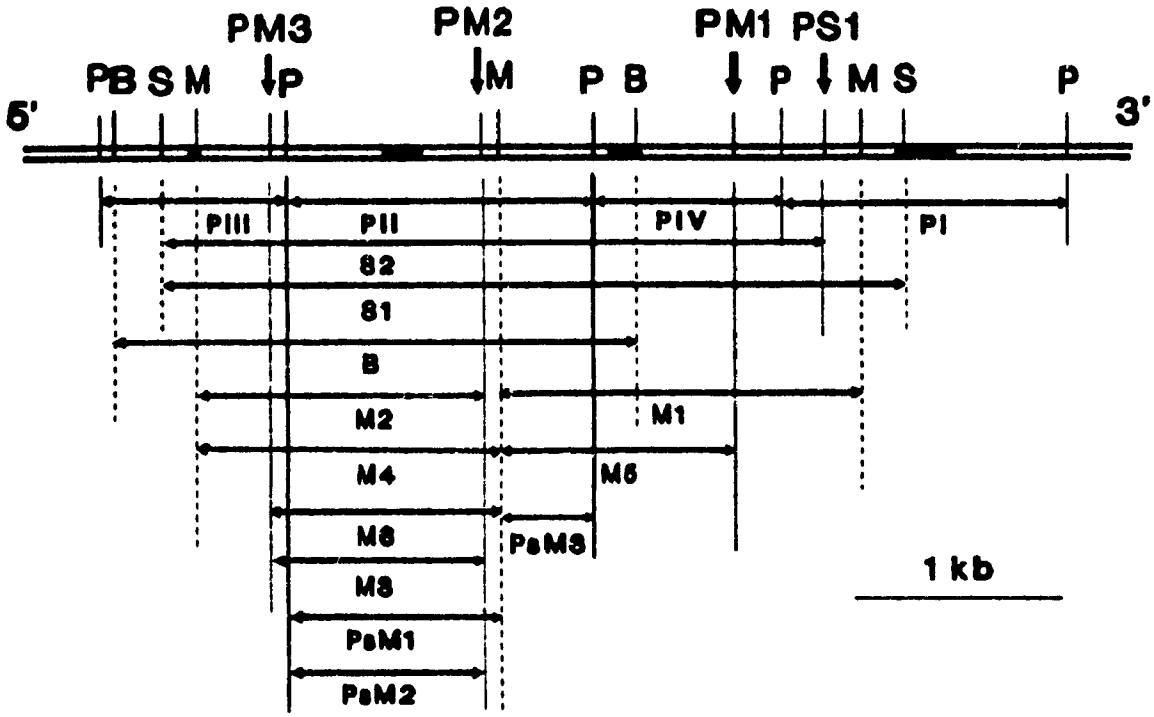

Fig. 16. The restriction map of GH gene with PstI (P), BamHI (B), SacI (S) and MspI (M) digestion showing the polymorphic sites. Closed boxes are exons and open boxes are introns. PM1, PM2, PM3 and PS1 indicate MspI and SacI polymorphic sites, respectively.

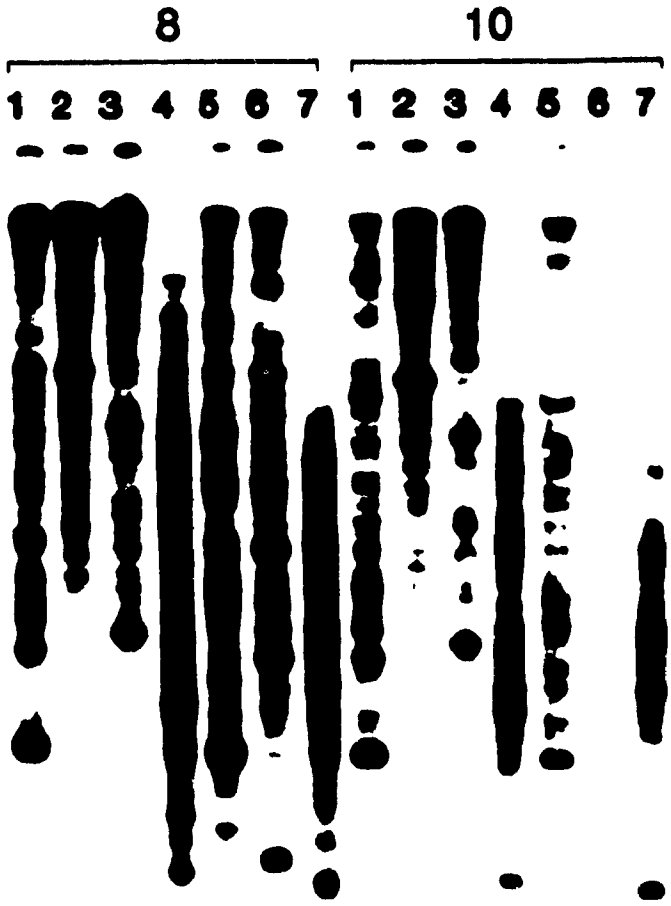

Fig. 17. Restriction enzyme analysis of two chickens of the fat line (#8 and #10) hybridized with PI. In lanes 1, 2, 3, 4 DNA was digested with PstI, BamHI, SacI or MspI, respectively. In lanes 5, 6 and 7 DNA was double digested with PstI and BamHI, SacI and MspI, respectively.

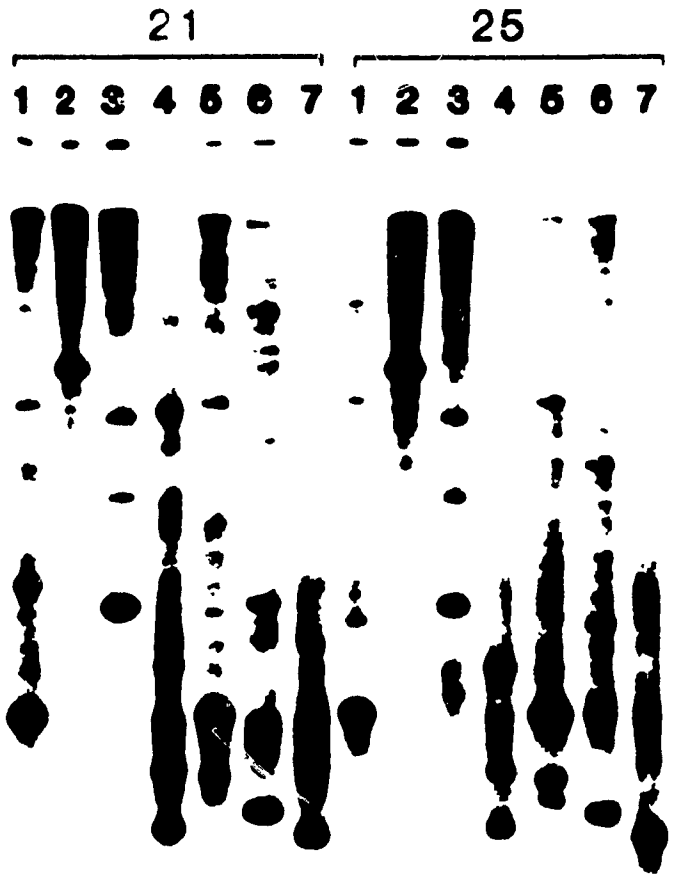

Fig. 18. Restriction enzyme analysis of two chickens of the lean line (#21 and #25) hybridized with PI. In lanes 1, 2, 3, 4 DNA was digested with PstI, BamHI, SacI or MspI, respectively. In lanes 5, 6 and 7 DNA was double digested with PstI and BamHI, SacI and MspI, respectively.

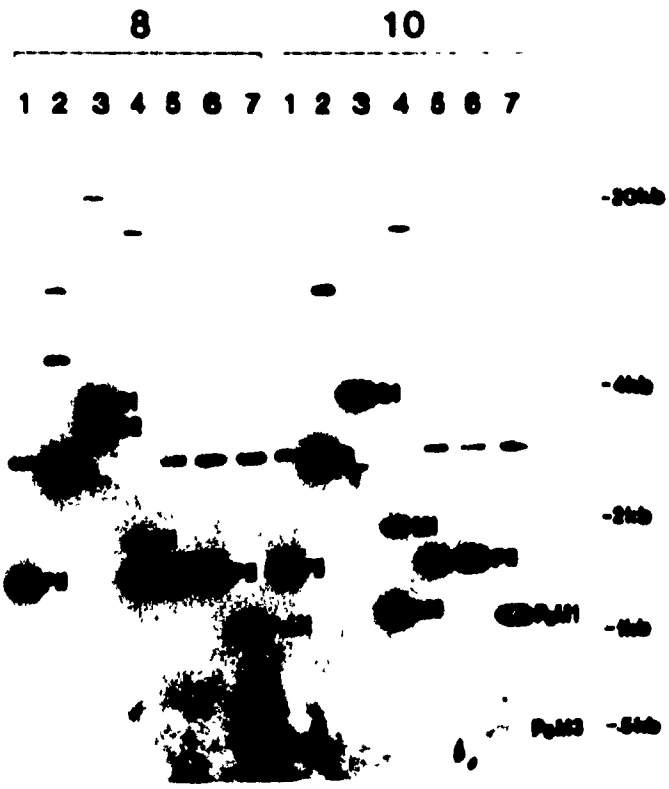

Fig. 19. Restriction enzyme analysis of two chickens of the fat line (#8 and #10) hybridized with PII. In lanes 1, 2, 3, 4 DNA was digested with PstI, BamHI, SacI or MspI, respectively. In lanes 5, 6 and 7 DNA was double digested with PstI and BamHI, SacI and MspI, respectively.

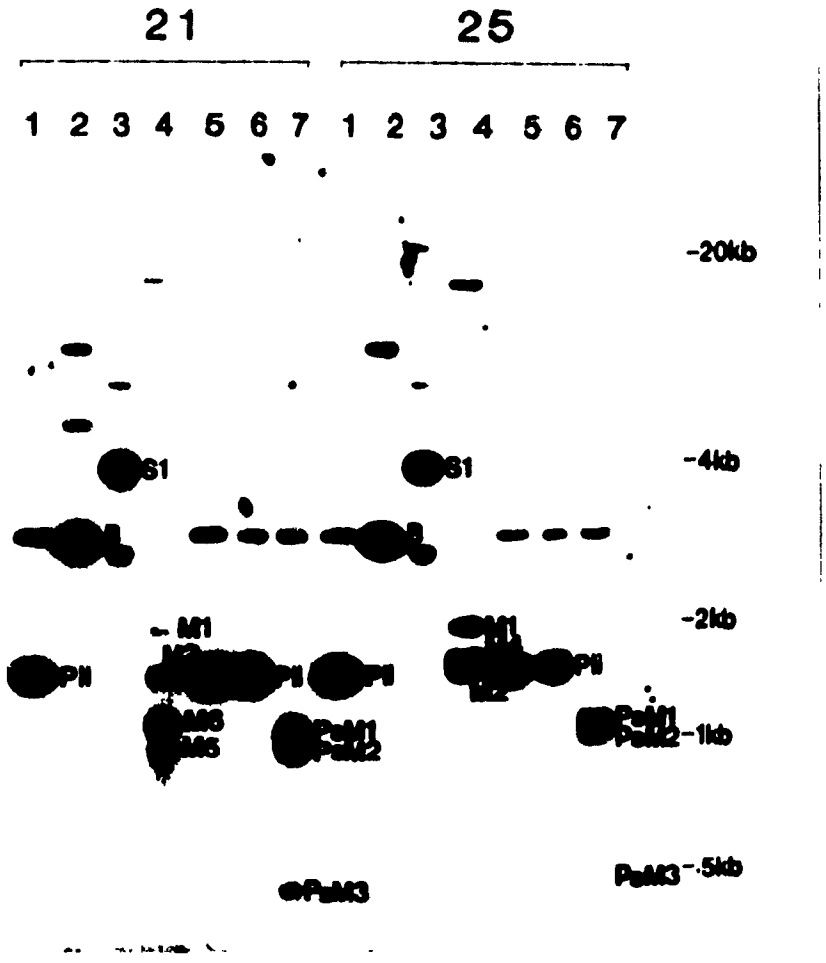

Fig. 20. Restriction enzyme analysis of two chickens of the lean line (#21 and #25) hybridized with PII. In lanes 1, 2, 3, 4 DNA was digested with PstI, BamHI, SacI or MspI, respectively. In lanes 5, 6 and 7 DNA was double digested with PstI and BamHI, SacI and MspI, respectively.

2.3. Assay for the GH-RFLP PM3 using the polymerase chain reaction

In this study, a PCR-based method to detect GH gene RFLPs was developed. Two primers (PM3F, PM3R) for amplification of a 627 bp fragment which contained the polymorphic site PM3 were designed on the basis of the chicken GH gene sequence (Tanaka *at al.*, 1992). In addition to the polymorphic site PM3, the amplified segment contained a second MspI site which was not polymorphic (Fig. 16). It was therefore expected that digestion of the PCR product with MspI would produce two or three fragments, depending on the genotype. The -/- homozygote (absence of an MspI restriction site at PM3) would be expected to produce two fragments of 284 bp and 343 bp. The +/+ homozygote (presence of an MspI restriction site at PM3) would be expected to produce three fragments of 284, 218 and 125 bp. The +/- heterozygote would be expected to produce four fragments of 284, 343, 218 and 125 bp (Fig. 21).

Fig. 22 shows the amplified PCR products and their MspI digests for three chickens of the +/+, +/- and -/- PM3 genotypes, respectively. The results were as expected by from the mapping studies of Fotouhi *et al.* (1993).

However, based on the GH gene sequence of Tanaka *et al.* (1992), the size of PCR amplified fragment should have been 627 bp, and not 840 bp as in our determination. In particular, one of the MspI fragments, fragment B (Fig. 22), was about 200 bp longer than the sequence expected from Tanaka's chicken GH gene sequence. This additional sequence was located in the first intron, which in the chickens analyzed here may be larger than in the chicken analyzed by Tanaka *et al.*

(1992).

The second pair of primer PM1F and PM1R was expected to amplify a 690 bp fragment which contains the polymorphic site PM1 in intron 4. No amplification was observed using different annealing temperatures and formamide concentrations (52, 54, 56, 58 and 60^{0}C with 5% formamide and 54^{0}C with 0, 1%, 2% and 3% formamide), different concentration of template DNA (100, 75 and 50 ng in 50 μl reaction volume), different concentrations of primer (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 μM in 50 μl reaction volume), different concentrations of magnesium (1.5, 1.7, 1.8, 1.9, 2.0 and 2.1 mM final concentration) and different annealing times (1 and 2 minutes). Pre-digestion of genomic DNA with NotI (which does not cut the GH gene) and BamHI restriction enzyme (which does not cut the region to be amplified) did not improve the PCR amplification and multiple fragments were still amplified. Comparison of the primer with Tanaka's sequence by computer, indicated that PM1F primer has 7 bp (CCTTTCT) at the 5' end with were present in two repeat region in the second intron.

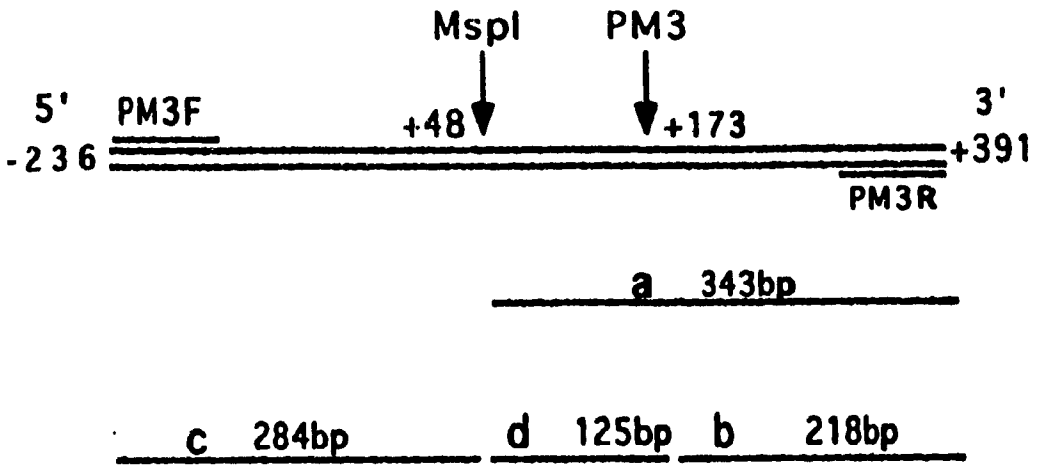

Fig. 21. Expected PCR product and its MspI digests. Two primers (PM3F, PM3R) were designed for amplification of a 627 bp fragment which contained the polymorphic site (PM3) and non-polymorphic MspI site (MspI). The arrows indicate the position of the MspI site. Digestion of PCR product with MspI would produce two (a and c), three (b, c and d) or four fragments (a, b, c and d) for genotype -/-, +/+ and +/-, respectively.

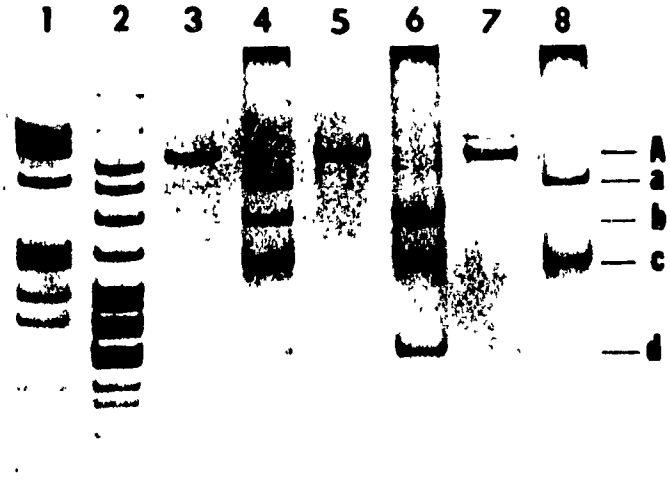

Fig. 22. Analysis of the PM3 genotype by PCR and MspI digestion. The PCR products were separated by 7% PAGE and visualized by DNA silver staining. Lane 1 and 2 are the molecular weight markers (ϕX-174-RF DNA HaeIII digest and pBR322 DNA-MspI digest). Lane 3, 5 and 7 are undigested PCR products. Lane 4, 6 and 8 are PCR products digested with MspI restriction enzyme. The genotype were +/- (lanes 3 and 4), +/+ (lanes 5 and 6) and -/- (lanes 7 and 8).

IV. Restriction Fragment Length Polymorphisms Associated with Production Traits Revealed by Random cDNA Probes

1. Introduction

Restriction fragment length polymorphisms can be detected by using minisatellites, genomic DNA or cDNA as probes. Minisatellite probes consisting of tandem repeats of core sequences shared by some of these regions detect polymorphisms at many loci (Jeffreys *et al.*, 1985a). Genomic DNA and cDNA probes can be used to detect polymorphic sites within specific genes (Litt and White, 1985). The difference between a genomic DNA clone and cDNA clone from the same gene is that cDNA clone does not contain intervening sequences (introns). Furthermore, if the genomic DNA clone used as a probe contains a repeated sequence, it will hybridize to many areas of the genome and may make RFLP analysis impossible. In the first part of my study, MspI polymorphism in a known gene, GH gene, was studied. In this part, polymorphisms in unknown genes were explored.

The production traits we analyzed were body weight (BW), age at sexual maturity measured as the age at first egg (AFE), egg production and Marek's disease resistance. In this study, the genomic DNA of chickens from strain 7, 8, 9, 8R, S and K (Gavora, 1979; Gavora *et al.*, 1986; Gavora and Spencer, 1983; Kuhnlein *et al.*, 1989) were used to detect RFLP with cDNA probes. In order to save time, DNA from 15 individuals of each strain were pooled. These strain pools were run on one gel together with a random individual of each strain and then hybridized to each

probe. Differences in the DNA band pattern or band intensity between pools or between pool and individuals indicate the presence of polymorphisms.

The recognition sequence of MspI contains the dinucleotide CpG which is highly mutable. MspI restriction enzyme therefore gives rise frequently to RFLPs (Cooper and Schmidtke, 1984). Barker *et al.*, (1984) estimated that in the human population, one out twenty MspI sites are polymorphic. DNA polymorphisms revealed by MspI digestion have therefore been used to diagnose human genetic disease (Cooper and Schmidtke, 1984).

The analysis of Fotouhi et al. (1993) and my own study (see chapter III in this thesis) revealed several MspI polymorphisms in the chicken growth hormone gene. It was therefore reasonable to choose this enzyme in our search for RFLPs detected by anonymous cDNA clones.

Production traits are controlled by multiple genes, each gene contributing a little to the trait performance. Mutations arising from base substitutions, insertions, deletions, translocations or rearrangements may alter the gene and protein expression. My rationale was to search for polymorphic sites in random genes and then correlate the polymorphisms to production traits. If a polymorphism is found to be associated with a trait, there are two possibilities, (1) the polymorphism has a direct effect on the production trait due to an alteration of the gene expression or the gene product or (2) the polymorphism is linked to a mutation which affects the production trait. In either case, the polymorphism can be used in marker-associated selection.

2. Material and Methods

2.1. Strains of chicken

The chicken strains 7, 8, 9, 8R, S, and K were used in this study. Strains 7, 8, 9 and 8R were derived from four North American commercial stocks of White Leghorns. Strain 7 was kept non-selected by random mating since 1958. Strains 8 and 9 were derived from strain 7 in 1969 and selected for egg production and related traits (Gowe and Fairfull, 1984). Strain 9 was selected for high egg production rate from the first egg to 273 days of age and Strain 8 was selected for high number of eggs produced per hen housed from the first egg to 273 days of age. Between 1969-1976, inbred lines were developed from strains 8 and 9 by selecting the best females based on their performance from the best sires and dams of a large population of birds per strain. After 3 generations of selection, strain 8R was derived from the 10 best inbred lines of strains 8 and 9 in 1979 (Gavora, 1979; Gavora *et al.*, 1986; Gavora and Spencer, 1983).

Strain S and K are two White Leghorn strains, which were derived from a common genetic base (Kuhnlein *et al.*, 1989) at Cornell in 1936 and were kept as closed breeding populations, except that a few commercial Kimber chickens were inter-mated with strain K in 1936 and 1940. Since 1936, strain K has been selected for resistance to Marek's disease and high egg production, whereas strain S has been selected for susceptibility to Marek's disease. The two strains were introduced to the Animal Research Centre of Agriculture Canada (Ottawa) in 1966 and were since maintained by random mating without selection.

2.2. cDNA library

A cDNA library was purchased from Stratagene. The library had been made from broiler-breeder chicken liver RNA cloned into Uni-ZAP^{TM} XR (Lambda ZAP^{R} II vector). Briefly, the vector was digested with EcoRI/XhoI and treated with Calf Intestinal Alkaline Phosphatase (CIAP) and the cDNA was synthesised from mRNA by using reverse transcriptase. The single-stranded termini of the double-stranded cDNA were digested with T4 DNA polymerase and EcoRI adaptors were ligated to the blunt ends. Followed by XhoI digestion which released the EcoRI adaptor and residual linker primer from the 3'-end of the cDNA. The released linker fragments were separated from the cDNA by Sepharose CL-4B chromatography. The cDNA was then precipitated and ligated to the Uni-ZAP XR vector arms. The vector size was about 40.8 kb. The average insert size of cDNA was about 1 kb. The lambda library was packaged using Gigapack^{R} II Gold packaging extract and plated on the *E.coli* cell line PLK-F'.

2.3. Isolation of random probes from cDNA library

2.3.1). Preparation of indicator bacteria

The host strain XL1-Blue cells had been shipped as glycerol stock in NZY media. Bacteria were removed with a sterile wire loop from a frozen (-70^{0}C) stock and streaked onto an NZY agar plate (one litre NZY broth contains 5 g Nacl, 2 g $MgSO_4.7H_2O$, 5 g Yeast Extract, 10 g NZ Amine and adding 15 g Difco Agar). After incubation at 37^{0}C overnight, a liquid culture in LB media supplemented with 0.2%

maltose and 10 mM $MgSO_4$ was inoculated with a single colony. The culture was grown overnight with vigorous shaking at 30^0C . The bacteria were pelleted by centrifugation at 1000 xg for 10 minutes and then gently resuspended in 0.5 volumes of 10 mM $MgSO_4$.

2.3.2). Plating

The cDNA library was titrated by using the following steps. Ten μl of the packaged phages from the library were diluted with 990 μl of SM buffer (5.8 g NaCl, 2 g $MgSO_4$, 50 ml 1 M Tris-Cl, pH 7.5, and 5 ml 2% gelatin per litre). From this stock solution a series of 1:10 dilution was made with SM buffer until the original library was diluted 10^6 fold. Ten μl of each titration was plated with 200 μl of $O.D._{600}$ = 0.5 XL1-Blue cells.

Plating was carried out by following the ZAP-cDNA^{TM} synthesis kit protocol (Stratagene). In brief, phages and XL1-Blue cells were pre-incubated at 37^0C for 15 minutes with gently shaking, then 2.5 ml of prewarmed (48^0C) top agar (NZY broth with 0.7% agarose) were added and poured immediately onto a prewarmed (37^0C) NZY agar plate. The plates were incubated overnight at 37^0C.

Single plaques were isolated by coring with a Pasteur pipette and suspending the core in 0.5 ml SM buffer, containing a drop of chloroform. The titre of a single plaque suspension was about 2.5 x 10^5 phages/ml.

2.3.3). Isolation of lambda phage DNA

In order to isolate insert-containing lambda phage DNA, 200 μl of a single plaque suspension were plated with 600 μl indicator bacteria on a plate (150 x 15 mm). After incubation overnight at 37^{0}C, 7 ml isolation buffer (10 mM Tris pH 7.5, 10 mM $MgSO^4$) was added and the plate gently shaken on a shaking platform at room temperature for 1.5 hours. The isolation buffer was collected from the plate into a 15 ml tube, and centrifuged for 10 minutes at 4000 xg in a bench top centrifuge. The supernatant (about 6 ml per tube) was transferred to a new tube. Two μl of DNase (1 mg/ml) were added to the supernatant. The tube was incubated at 37^{0}C for 15 minutes with shaking to digest bacterial genomic DNA. One volume of 20% PEG 8000, 2 M NaCl, 10 mM Tris and 10 mM $MgSO_4$ was added. After one hour at 0^{0}C the tubes were centrifuged at 10^4 xg for 10 minutes. The pellet was suspended with 0.5 ml of TE buffer containing 0.1 M NaCl. The DNA was extracted twice with phenol/chloroform (1:1 v/v) and once with chloroform and precipitated with two volumes of 100% cold ethanol. The pellet was washed twice with 70% ethanol, air dried and dissolved in 60 μl TE buffer containing 20 μg/ml RNase. The concentration of isolated DNA was approximately 200 μg/ml as estimated by ethidium bromide staining and comparison with a molecular marker in an agarose gel.

2.3.4). Isolation of the insert DNA for probes

Lambda phage DNA (about 1.2 μg) was double digested with restriction enzyme SacI and XhoI by first incubating the DNA with 15 units of SacI in 1x One-Phor-All

buffer. The reaction volume was 40 μl. After incubation at 37°C for 2 hours, 15 units of XhoI and 2x One-Phor-All buffer (final concentration) were added (total volume 60 μl) and incubation was continued at 37°C overnight.

The insert was separated from vector by electrophoresis in a 0.8% low-melting temperature agarose gel at 50 V for 1.5 hour. The gel was stained with ethidium bromide and the DNA bands visualized under UV light. The insert band was cut out by using a razor blade (Fig. 23). The gel slice containing the insert band was weighed and 3 ml of H_2O was added for each gram of gel. The tube was heated at 65°C until the gel was completely melted. The homogenized DNA/agarose solution was stored at -20°C and used for probe labelling.

2.3.5). *In vivo* excision of phagemid

Phagemid DNA was prepared by following the *in vivo* excision protocol of Stratagene. In brief, a plaque was cored from an agar plate and transferred into a sterile microcentrifuge tube containing 0.5 ml of SM buffer and 20 μl of chloroform. The tube was vortexed and incubated at room temperature for 1 to 2 hours.

Two hundred μl of XL1-Blue cells (OD_{600} = 1.0, pre-selected for the F' episome by growth on LB/tetracycline agar plates) were infected with 200 μl of the single plaque phage stock (containing >1 x 10^5 phage particles) in a 15 ml tube, and 1 μl of R408 helper phage (>1 x 10^6 pfu/ml; Stratagene) was added. The mixture was incubated at 37°C for 15 minutes. After adding 5 ml of 2x YT broth (10 g NaCl, 10 g Yeast Extract and 16 g Bacto-Tryptone per litre) the tube was further incubated

at 37^{0}C for 3 hours with shaking. The cultured bacteria were heated at 70^{0}C for 20 minutes, and then centrifuged at 4000 xg for 5 minutes. The supernatant was decanted into a sterile tube. This stock contained the pBluescript phagemid packaged as filamentous phage particle. Bacterial cells incubated with R408 helper phage alone without recombinant Uni-ZAP XR phage were used as a negative control.

An aliquot of 200 μl of XL1-Blue host cells was infected with 200 μl of phagemid stock from the step above in a 15 ml tube and the tube was incubated at 37^{0}C for 15 minutes. Fifty μl of the cultured bacteria was plated on an LB/amp plate and incubated overnight at 37^{0}C. Colonies appearing on the plate contained the pBluescript double stranded phagemid with the cloned DNA insert. The bacteria infected with helper phage alone did not grow since they did not contain ampicillin resistance genes. Bacterial clones were further purified by replating on LB/amp plate and re-isolating single-colonies.

For the isolation of phagemid DNA, a single colony was picked into a 15 ml Conical tube with 4 ml LB media/Amp and incubated at 37^{0}C overnight. The phagemid DNA was then extracted by using the standard method for Mini-preparation of plasmid DNA (see III.1.2.). The insert was isolated by digesting the phagemid DNA with ScaI and XhoI, followed by gel-electrophoresis.

2.4. Southern blotting for detecting polymorphism

Chicken genomic DNA was extracted as described in III.1.6.1). The DNA pools for each strain were made by mixing equal amounts of DNA from 15 individuals of

each strain. DNA digestion, gel electrophoresis, Southern blotting, prehybridization and hybridization were the same as described in III.1.6.5).

2.5. Trait associations of RFLPs

To test the association between RFLPs detected with Probe #2, #5 and #9, two groups of individuals for each trait (housing body weight -- HBW and age at first egg -- AFE) were formed by taking the samples from two tails of the trait distribution in both strain 8 and strain 9. Each group consists of 15 individuals. The difference in frequency of a RFLP between two groups (high and low HBW or early and late AFE) was tested statistically by using a Chi-Square test (Siegel and Castellan, 1988).

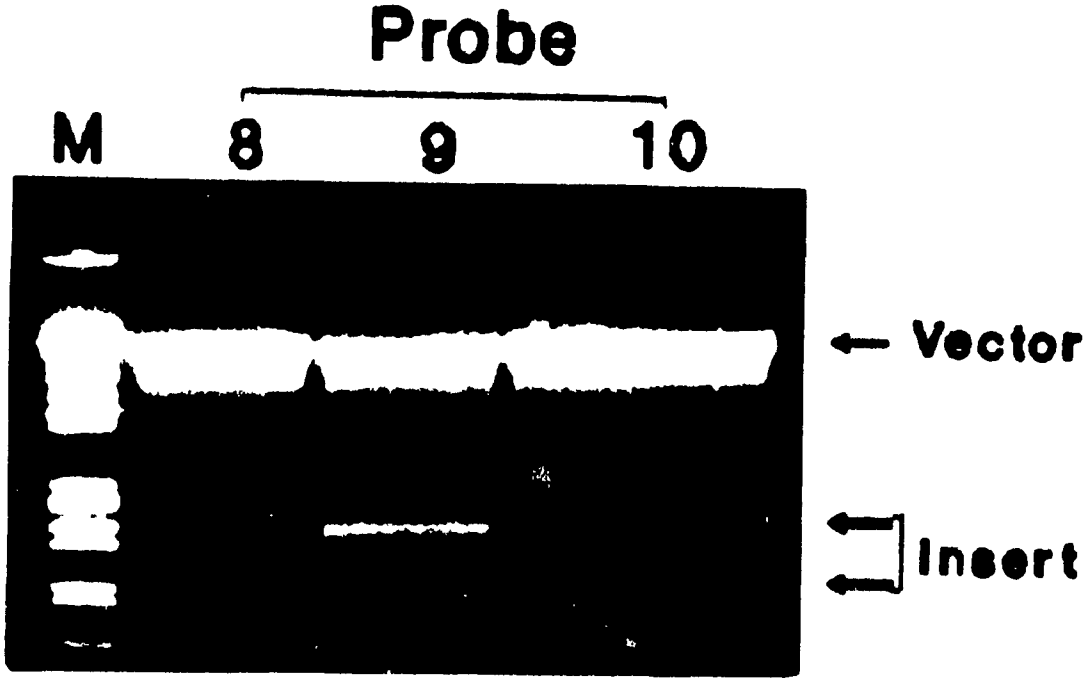

Fig. 23. The cDNA clones double digested with SacI and XhoI restriction enzyme. The fragments were separated by electrophoresis on a 0.8% LMT agarose gel. M is a molecular weight marker, and 8, 9 and 10 are lambda ZAP clones.

3. Results and Discussion

3.1. Detection of RFLP by using individual and pooled DNA

A total of six strains were analyzed for RFLPs by comparing "strain pools" and selected individuals by Southern blotting with 10 different random clones. Comparison of band intensities among pools and between pools and individuals revealed three different classes of probes, those which hybridized to repetitive DNA (Fig. 24), those which did not reveal RFLP's (Fig. 25) and those which showed RFLPs (Fig. 26, 27, 28). The results obtained with the 10 clones are summarized in Table 2.

Figure 26 shows the result from hybridization with Probe #2. In strains 8, 9 and 8R two bands (A_1 and A_2) were observed while strain 7 and all individuals only produced a single band (A_1 or A_2). Hence strains 8, 9 and 8R segregated for an RFLP. Since strain 7 is the un-selected control strain of strain 8, 9 and 8R, it is possible that A_1 had been co-selected with selection for egg production trait and disease resistance which was exercised in the latter three strains. Similarly, strain S segregated for A_1 and A_2 while strain K which had been selected for egg production traits and MD resistance is of the A_1 genotype.

The hybridization result with Probe #5 is shown in Fig. 27. Four bands were observed in all the pools. Two bands were present at equal intensity in all samples, while the other two bands (B_1, B_2) varied, indicating an RFLP. Four of the individuals sampled were B_2B_2, one was B_1B_1 and one was B_2B_1. Among the strains studied, all showed the same relative band intensities except 8R, where B_1 was less intensive indicating that the frequency of B_1 in strain 8R was low. For the other

strains, there was no indication that the selection affected the frequency of alleles.

Probe #9 (Fig. 28) revealed polymorphisms in strain 7, 8, 9 and 8R, but not in strain S and strain K. Further, segregation in strain 7, 8, 9 and 8R was about equal, indicating that the RFLP frequencies were not affected by selection.

3.2. Analysis of trait-associated RFLPs with Probe #2, #5 and #9

The bulk analysis of strains showed that there were polymorphisms in the genes represented by the cDNA probes #2, #5 and #9 and that the selection for egg production traits and Marek's disease did affect the frequency of some of these alleles. We questioned whether any of these polymorphisms revealed by probe #2, #5 and #9 were associated with one or more of the production traits which were subject to selection. Two production traits, housing body weight (HBW) and age at first egg (AFE), were studied in strain 8 and strain 9. For each trait, two groups of chickens were analyzed, representing the two tail-ends of the trait distribution. Each group containing 15 individuals was analyzed for RFLPs with probe #2, #5 and #9.

Table 3 and 4 show comparison of the frequency of different genotypes between high and low HBW and between early and late AFE. Here, A_1 and A_2 denote the alleles revealed by hybridization with probe #2, B_1 and B_2 with probe #5, and C_1 and C_2 with probe #9. The difference in frequency of an allele between high and low HBW, and between early and late AFE were tested by chi-square test (Siegel and Castellan, 1988).

Table 2. Summary of the hybridization results with ten cDNA probes[1]

Probe	Polymorphisms in different strains					
No.	7	8	9	8R	S	K
1	N	N	N	N	N	N
2	N	Y	Y	Y	Y	N[2]
3	N	N	N	N	N	N
4	N	N	N	N	N	N
5	Y	Y	Y	Y	Y	Y
6	N	N	N	N	N	N
7	N	N	N	N	N	N
8	N	N	N	N	N	N
9	Y	Y	Y	Y	N	N
10	N	N	N	N	N	N

[1] Y = polymorphic and N = not polymorphic.

[2] Fixed for the opposite allele.

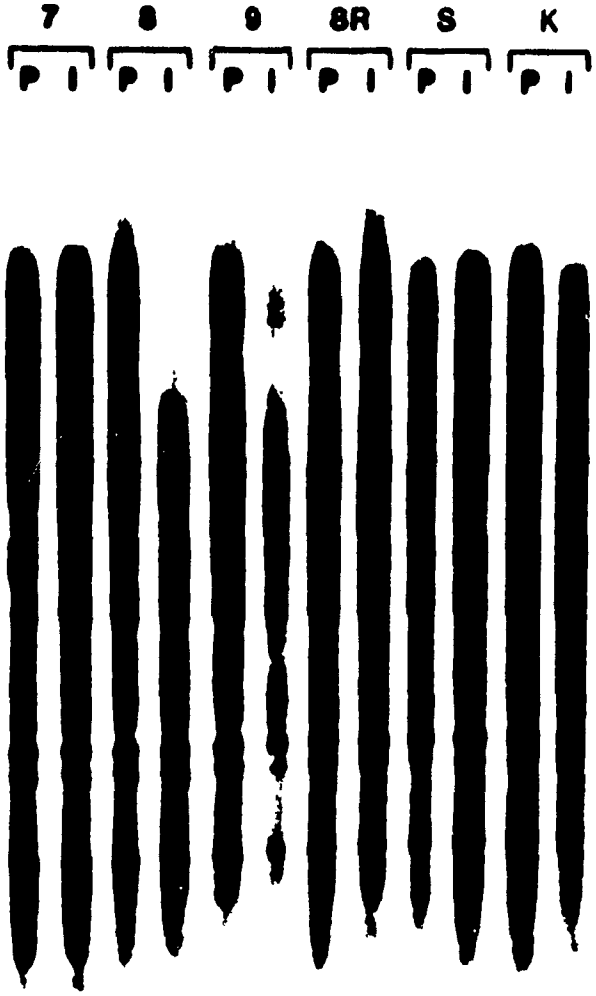

Fig. 24. Southern blot of pools of genomic DNA (P) and genomic DNA from individuals (I) hybridized with the cDNA probe #1, revealing repetitive DNA. The DNA was digested using MspI and the various strains are indicated.

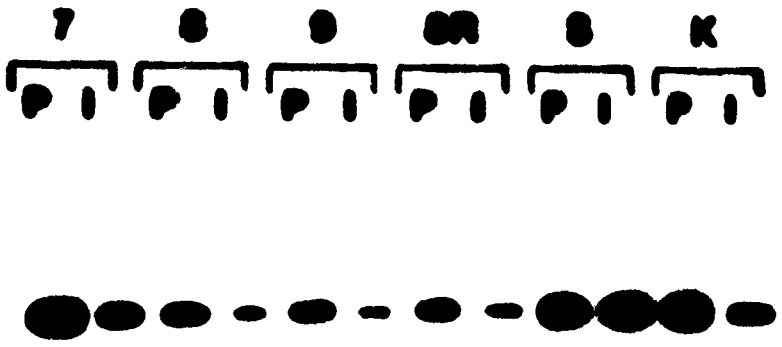

Fig. 25. Southern blot of pools of genomic DNA (P) and genomic DNA from individuals (I) hybridized with the cDNA probe #7, revealing monomorphic DNA. The DNA was digested using MspI and the various strains are indicated.

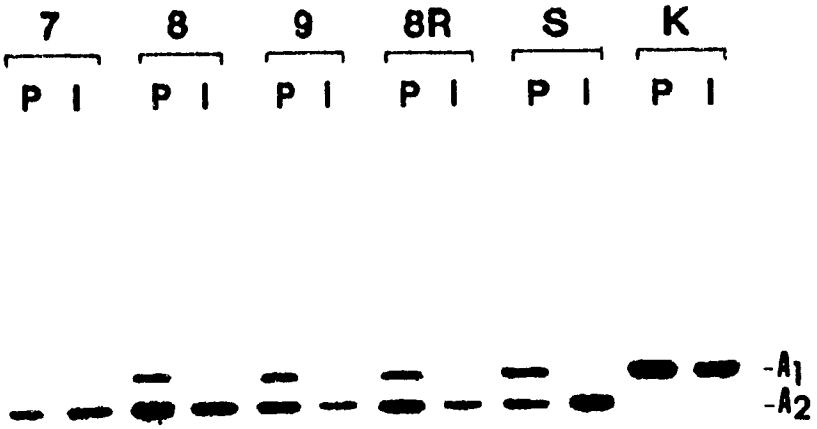

Fig. 26. Southern blot of pools of genomic DNA (P) and genomic DNA from individuals (I) hybridized with the cDNA probe #2. The DNA was digested using MspI and the various strains are indicated. MspI polymorphisms are present in strains 8, 9, 8R and S.

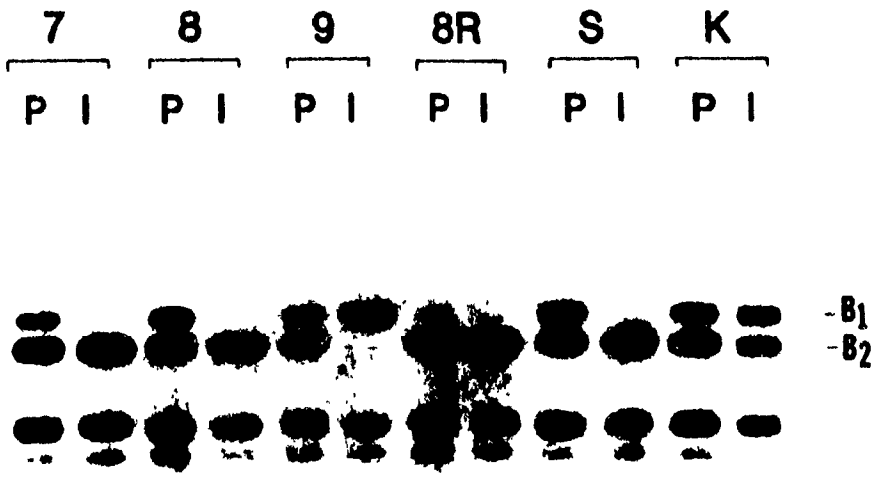

Fig. 27. Southern blot of pools of genomic DNA (P) and genomic DNA from individuals (I) hybridized with the cDNA probe #5. The DNA was digested using MspI and the various strains are indicated. MspI polymorphisms are present in strains 7, 8, 9, 8R and S.

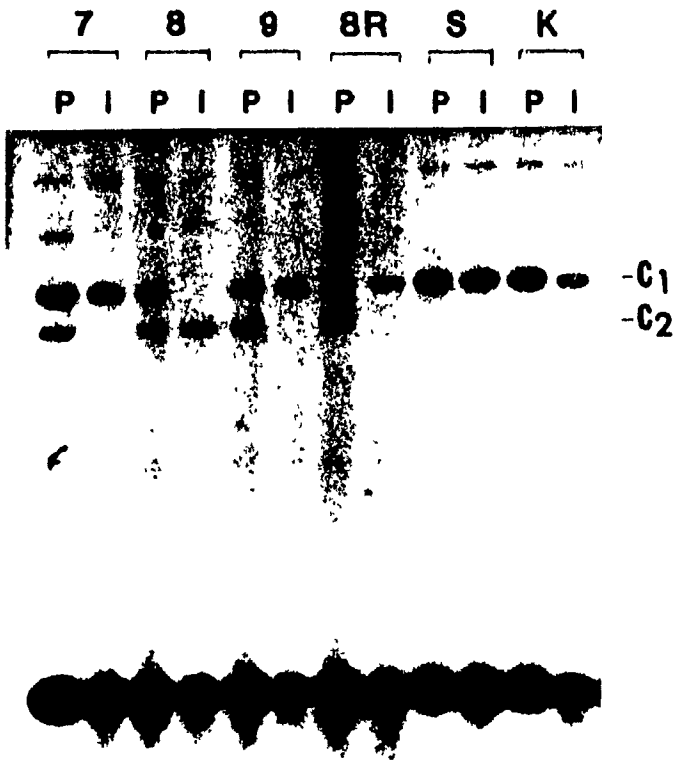

Fig. 28. Southern blot of pools of genomic DNA (P) and genomic DNA from individuals (I) hybridized with the cDNA probe #9. The DNA was digested using MspI and the various strains are indicated. MspI polymorphisms are present in strains 7, 8, 9 and 8R.

The results are shown in Table 3 and 4. The average frequency of A_2 allele was 0.86 in strain 8 and 0.90 in strain 9. Statistical analysis by X^2 test indicated that the differences of the frequency of the A_2 allele between high and low HBW and between early and late AFE in both strains were not significant at $P \leq 0.10$.

With probe #5, the average frequency of B_2 allele was 0.76 in strain 8 and 0.75 in strain 9. There was a trend towards an increase of the B_2 allele in the group of higher HBW and earlier AFE, respectively. However the differences were not significant at $P \leq 0.10$ except between high and low HBW in strain 8.

The average frequency of C_2 allele was 0.45 in strain 8 and 0.48 in strain 9. There were no significant differences at $P \leq 0.10$ between high and low HBW, between early and late AFE in both strains.

With Probe #9, only the C_1 or the C_2 allele was observed in each individual, indicating the absence of heterozygotes. Fig. 29 shows an example of 20 individuals sampled from strain 8, ten from the late AFE group and ten from the high HBW group. It indicates that the gene which encodes the cDNA #9 is present in a haploid state. It raised the possibility that the gene was located on the sex chromosome, since all the individuals studied were females and the sex chromosome pattern of bird is opposite to mammals (male: ZZ and female: ZW). A gene located on either of the sex chromosomes, would be present in a single copy in females. In males the gene would be absent if located on the W chromosome, and present in a diploid state if located on the Z chromosome. In the latter case we would expect to observe heterozygotes among the males. To test this hypothesis, 85 individuals from strain 7,

68 females and 17 males, were analyzed. Among the males 5 C_2 and 12 C_1 genotype were observed and among females 26 C_2 and 42 C_1 genotypes were observed. Thus, frequency of C_2 allele among males is 0.294, and among females is 0.382. Heterozygotes were again absent among the males. Thus the gene which codes for cDNA #9 is present in haploid state and is not located on the sex chromosome.

We further investigated whether this haploid genetic element is inherited from the female, the male or at random. To answer these questions, a segregation analysis of the probe #9 polymorphism was conducted in 13 families consisting of 6 male parents, 13 female parents and 95 offspring. The results are presented in Table 5. When both parents had the C_2 genotype, all offspring were C_2; and when both parents were C_1, all offspring were C_1 genotype. Thus the genotypes of the parents were stably inherited. In matings where the genotypes of the two parents differed, all the offspring inherited the maternal genotype. There was a single exception and we should check this offspring with its parents by using DNA fingerprinting to verify whether the individual was indeed the offspring of the particular parents. The result indicate that similar to mitochondria, the offspring's genotype were determined by the female parent. However, at this stage, we can not exclude the possibility that this genetic element comes from mitochandria DNA.

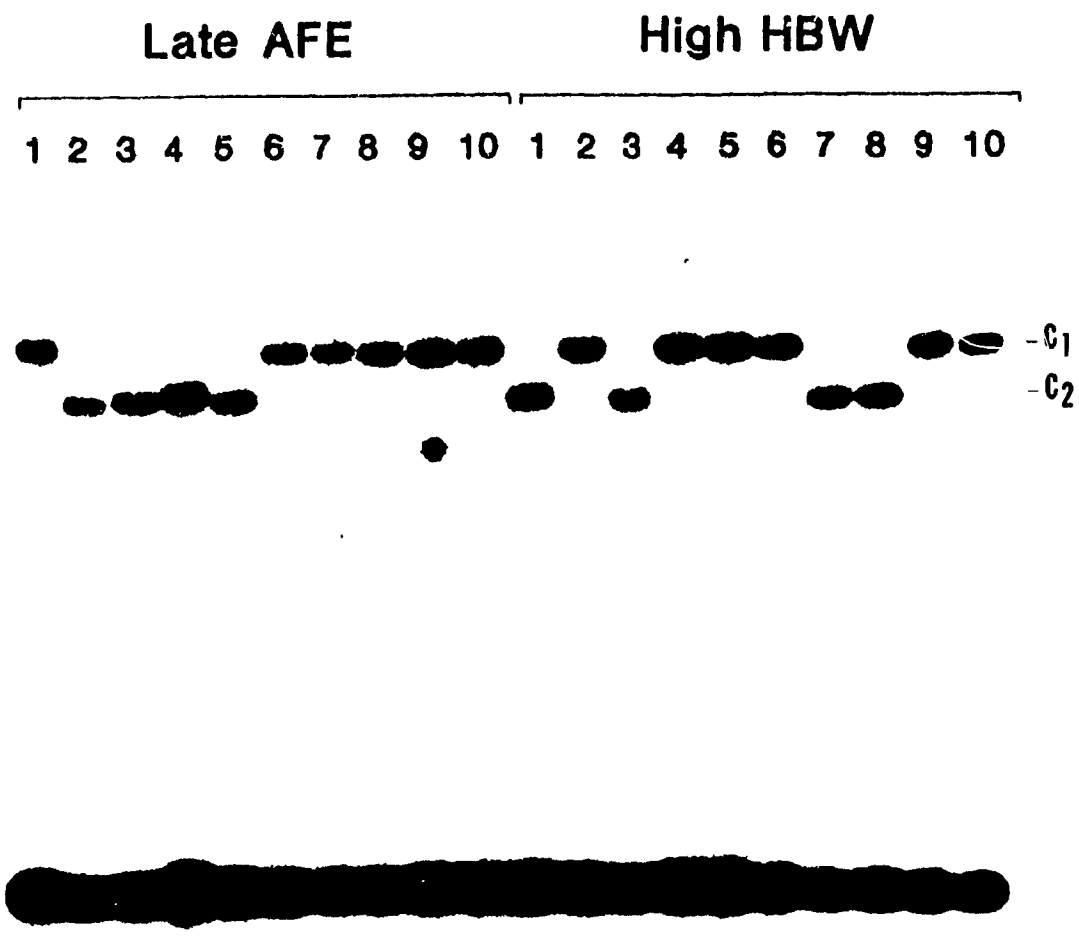

Fig. 29. RFLPs revealed by probe #9 in 20 individuals of strain 8. The first 10 individuals from late AFE and the second 10 individuals from high HBW. No heterozygous genotypes have been found. Fragments C_1 and C_2 indicate two genotypes.

Table 3. Frequency of genotypes at three anonymous loci in chickens with high and low housing body weight, respectively

	Genotype	Strain 8		Strain 9	
		High	Low	High	Low
	A_1/A_1	0	0	0	1
Probe #2	A_1/A_2	3	5	4	0
	A_2/A_2	12	9	11	14
Frequency	of A_2	0.90	0.82	0.87	0.93
	B_1/B_1	0	2	0	1
Probe #5	B_1/B_2	3	4	6	5
	B_2/B_2	12	9	9	9
Frequency	of B_2	0.90	0.73*	0.80	0.77
	C_1/C_1	8	10	5	7
Probe #9	C_1/C_2	0	0	0	0
	C_2/C_2	7	4	10	8
Frequency	of C_2	0.47	0.29	0.69	0.53

* Significantly different at $p \leq 0.10$ between high and low housing body weight

Table 4. Frequency of genotypes at three anonymous loci in chickens with early and late onset of egg-laying, respectively

	Genotype	Strain 8		Strain 9	
		Early	Late	Early	Late
	A_1/A_1	0	2	0	0
Probe #2	A_1/A_2	2	2	3	3
	A_2/A_2	12	11	12	12
Frequency	of A_2	0.93	0.80	0.89	0.90
	B_1/B_1	1	2	0	2
Probe #5	B_1/B_2	5	6	6	7
	B_2/B_2	8	7	8	6
Frequency	of B_2	0.75	0.67	0.79	0.63
	C_1/C_1	7	7	9	10
Probe #9	C_1/C_2	0	0	0	0
	C_2/C_2	7	8	5	5
Frequency	of C_2	0.50	0.53	0.36	0.33

Table 5. Segregation of probe #9 polymorphisms in strain 7

Parent Genotype		Offspring Genotype	
Father	Mother	C_2	C_1
C_2	C_2	15	0
C_1	C_1	0	32
C_2	C_1	0	25
C_1	C_2	22	1

V. Conclusion

Advances in molecular biology have provided and will continue to provide new tools and methods which can be used in animal breeding. In the next century, two research areas will be of particular importance and will bring revolutionary changes in animal breeding. These two areas are development of techniques to identify and characterize genes and the development of gene transfer. In this study, the focus was on the first area, to identify genes or genetic markers which are associated with production traits.

Two approaches were explored in the present study, (1) localizing polymorphisms in a known gene, the GH gene, and developing a rapid method to detect these polymorphisms; and (2) searching for polymorphisms in unknown genes using cDNA clones isolated randomly from a chicken cDNA library as probes.

In the first approach, we want to develop a efficient method to detect polymorphisms in the chicken GH gene. Traditionally, restriction fragment length polymorphism analyses are conducted by DNA Southern blotting. The limitation of using DNA Southern blotting in animal breeding is that it is time-consuming and laborious, especially when large number of samples are analyzed. We explored an alternative method, the polymerase chain reaction (PCR), and traditional Southern blotting. There are two advantages of using PCR instead of Southern blotting: 1) A PCR detection only takes a few hours to finish while Southern blotting may take a several days; 2) PCR can be automated, allowing large number of samples to be

rapidly analyzed with relatively little labour. The only pre-requisite is that the sequence of the regions flanking the polymorphic site has to be known for designation of PCR primers. The solution is either to study polymorphisms in known genes, whose sequences is published or to isolate the gene of interest, localize the polymorphisms in the gene and sequence regions flanking the polymorphic sites. The MspI polymorphisms of GH gene were mapped by Fotouhi *et al.* (1993) in the approximate position of the gene by using a turkey GH cDNA. However, to develop a PCR assay for these polymorphisms, the sequences flanking the polymorphic sites had to be determined. We therefore had to isolate the chicken GH genomic gene and sequence the gene.

After screening about 50,000 colonies from a genomic library, a cosmid clone containing the GH gene was isolated. The GH gene was subcloned into the plasmid pUC18 and about 2200 bp were sequenced. This sequence work was halted when Tanaka *et al.* (1992) published the genomic sequence of the chicken GH gene. Comparing our sequence with the chicken GH cDNA and the published genomic sequence indicated that we had found the correct clone. In addition, RFLPs detected at MspI and SacI sites using the genomic subclone PII were identical to those detected by Fotouhi *et al.* (1993) using cDNA as a probe.

Using the sequence information published, we developed PCR-based method for the MspI RFLP at the site PM3. The genotypes of individuals at this site determined with our PCR-based method were in agreement with Fotouhi's determinations by Southern blotting. The PCR-based method can be commercially adapted in marker-

assisted selection programs.

Surprisingly, when using primers based on the sequence information from Tanaka *et al.*, (1992) to amply the PM3 alleles, the amplified fragment was always 200 bp larger than predicted. This could be due to an insertion in the first intron of the GH gene, reflecting a difference between strains.

Application of molecular genetics to poultry breeding is limited due to the availability of informative genetic markers associated with production traits. Thus, scarching for new genetic markers is important. In the second part of the thesis, polymorphisms at MspI restriction sites were studied in 6 different strains of chickens by using ten random cDNA clones. Bulk analysis using pooled samples and one individual from each strain led to the rapid identification of those clones which reveal RFLPs. Among ten clones, three gave rise to RFLPs at MspI sites.

One MspI polymorphic site was detected with each probe. To investigate the association between production traits and the MspI polymorphism detected with the three cDNA probes, further studies were conducted in two strains for two traits, HBW a id AFE. However, no significant associations could be detected within the relatively small number of individuals.

To our surprise, the MspI polymorphism revealed by Probe #9 was present on a haploid genetic element. This could be the discovery of a new chromosome which is present in a haploid state. The segregation analysis in 13 families including 6 males, 13 females and 95 offspring showed that the inheritance of this polymorphism was stringently determined by the genotype of the female parent.

VI. References

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