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Isolation and Characterization of the Triiodothyronine Regulatory Region of the Fatty Acid Synthase Gene in Avian Species

Delfina Rivera Valencia

ISOLATION AND CHARACTERIZATION OF THE TRIIODOTHYRONINE REGULATORY REGION OF THE FATTY ACID SYNTHASE GENE IN AVIAN SPECIES

by

Delfina Rivera Valencia

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Chemistry

Western Michigan University
Kalamazoo, Michigan
August 1992

ISOLATION AND CHARACTERIZATION OF THE TRIIODOTHYRONINE REGULATORY REGION OF THE FATTY ACID SYNTHASE GENE IN AVIAN SPECIES

Delfina Rivera Valencia, M.S.

Western Michigan University, 1992

Fatty acids are utilized for energy storage in the form of triglycerides or neutral fats. One way to regulate fatty acid biosynthesis is to regulate the activity of fatty acid synthase (FAS), the enzyme that catalyzes the reaction. Previous studies have shown that regulation of FAS activity and synthesis is regulated by the hormone triiodothyronine, T_3, at the level of transcription. Thus, isolation and characterization of the promoter region of the FAS gene will enable the identification of the thyroid hormone response elements (TREs). This study could shed light on the mechanisms on how this gene can be controlled.

In this study we were able to: (a) isolate a putative FAS promoter region from chicken liver using known goose liver FAS promoter sequence and the polymerase chain reaction; (b) isolate, clone, and sequence the exon 3 of the chicken hypoxanthine phosphoribosyl transferase, HPRT, gene as a control from the experiments conducted in a; and (c) create deletions of a cloned 1.6 kb promoter region of goose liver FAS to determine the location of the T_3 response element present.

ACKNOWLEDGMENTS

I would like to extend my sincerest gratitude to the following:

Dr. Susan Stapleton, my adviser, for her wholehearted support and helpful advice throughout the entire project and in the preparation of this thesis;

Mr. Ken Rank, research associate from the Biological Sciences Department, for helping me refine my molecular biology techniques;

Dr. Pablito Nagpala, postdoctoral fellow at Upjohn Laboratories, for helping me with my sequencing reactions;

Dr. and Mrs. Ignacio and Milagros Valencia, my parents, for their unconditional love and support;

Professors, classmates, and friends who made my stay at WMU very meaningful and fruitful;

And last, but not least, to my Higher Power, who has always made all things work out for the best.

Delfina Rivera Valencia

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INTRODUCTION

Background Information

Fatty Acids

Fatty acids belong to a class of compounds that contain a long hydrocarbon chain with a terminal carboxylic group. A common example is palmitic acid, a naturally occurring 16-carbon fatty acid in biological systems (shown in Figure 1).

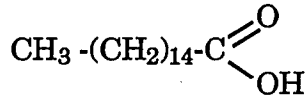


Figure 1. Chemical Structure of Palmitic Acid.

Other examples of naturally occurring fatty acids are lauric acid (a 12-carbon), myristic acid (a 14-carbon), and stearic acid (an 18-carbon). At physiological pH, the fatty acids are ionized and at that state they are referred according to their carboxylate form; example, palmitate.

Physiological Roles of Fatty Acids

Fatty acids are important in biological systems because of two reasons: (1) they the are building blocks of phospholipid and glycolipid which are important components of biological membranes, and (2) they are

fuel molecules which are utilized as energy storage for the body.

As fuel molecules, fatty acids are stored as triacylglycerides, which are otherwise known as neutral fats or triglycerides.

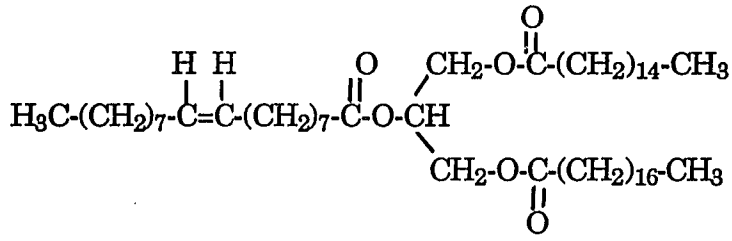


Figure 2. Structure of a Triacylglycerol.

Based on Figure 2, it can be deduced that triacylglycerols are highly reduced and anhydrous. As a consequence, complete oxidation of a fatty acid will yield about 9 kcal/g, whereas complete oxidation of a carbohydrate or a protein will give only 4 kcal/g.

The liver is the major site where fat biosynthesis and degradation occur. The oxidative degradation of fatty acids to yield fuel molecules occurs in the mitochondrial matrix. In contrast, synthesis of fatty acids occurs in the cytosol. The synthetic pathway is entirely distinct and consists of a set of reactions different from that of the degradative pathway. Understanding the synthesis of fatty acids is of critical importance to this study.

De Novo Synthesis of Fatty Acids

When food is taken in by the body, large molecules like

carbohydrates, proteins and fats are broken down into smaller units--glucose, amino acids, and fatty acids and glycerol, respectively. These are further degraded into a simpler unit that plays a central role in metabolism. Glucose and other sugars, amino acids, fatty acids and glycerol are converted into the acetyl unit of acetyl-CoA. Acetyl-CoA can be utilized in any of the following routes: The acetyl unit can be completely oxidized to CO_2 by the citric acid cycle with the subsequent production of ATP. Alternatively, three molecules of acetyl-CoA can form the six-carbon unit precursor of cholesterol and of ketone bodies. The third possible fate of acetyl-CoA is its export to the cytosol in the form of citrate for the synthesis of fatty acids.

Under conditions where acetyl-CoA and ATP are in high concentration (as in the fed state), the citrate leaves the mitochondria via the tricarboxylic acid (TCA) transport system and in the cytosol reacts with CoA and ATP to form acetyl-CoA and oxaloacetate, in a reaction catalyzed by citrate cleaveage enzyme.

mitochondrial acetyl-CoA + oxaloacetate $\longrightarrow$ citrate + CoASH

cytosolic citrate + ATP + CoA $\longrightarrow$ acetyl-CoA + oxaloacetate + ADP + P_i

Now in the cytosol, acetyl-CoA carboxylase activates acetyl-CoA into a three-carbon molecule called malonyl-CoA.

$$CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + ATP + HCO_3^- \longrightarrow {}^-OOC\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-SCoA} + ADP + P_i$$

acetyl-CoA malonyl-CoA

More of the acetyl-CoA further reacts with malonyl-CoA in the presence of NADPH to form the 16-carbon fatty acid called palmitic acid.

$$\text{acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ \longrightarrow \text{palmitate} + 7\ CO_2 + 6H_2O + 8\ \text{CoASH} + 14\ NADP^+$$

In higher animals, fatty acid synthesis involves only the formation of palmitate from which other saturated and monounsaturated fatty acids are formed.

The principal reactions and enzymes in fatty acid synthesis which were first elucidated in E.coli can be summarized in Table 1.

Fatty Acid Synthase

Table 1 shows that the de novo synthesis of fatty acids from acetyl CoA requires seven enzyme activities. In bacteria and plants, the enzymes exist as discrete monofunctional polypeptides. In animals, however, the enzyme activities are integrated into a single polypeptide chain--a multienzyme complex called fatty acid synthase (FAS). The multi-functional FAS is encoded by the FAS gene which is hypothesized to have been evolved by the fusion of several genes (Amy, Williams-Ahlf, Naggert, & Smith, 1992).

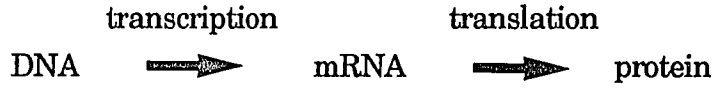
Table 1

Principal Reactions and Enzymes in Fatty Acid Synthesis
Elucidated in E.coli

Step	Reaction			Enzyme
1	Acetyl-CoA + HCO_3^- + ATP	$\longrightarrow$	malonyl-CoA + ADP + P_i + H^+	Acetyl-CoA carboxylase
2	Acetyl-CoA + ACP	$\rightleftharpoons$	acetyl-ACP + CoA	Acetyl transacylase
3	Malonyl-CoA + ACP	$\rightleftharpoons$	malonyl-ACP + CoA	Malonyl transacylase
4	Acetyl-ACP + malonyl-ACP	$\longrightarrow$	acetoacetyl-ACP + ACP + CO_2	Acyl-malonyl-ACP condensing enzyme
5	Acetoacetyl-ACP + NADPH + H^+	$\rightleftharpoons$	D-3-hydroxybutyryl-ACP + $NADP^+$	ß-Ketoacyl-ACP-reductase
6	D-3-hydroxybutyryl-ACP	$\rightleftharpoons$	crotonyl-ACP + H_2O	3-Hydroxyacyl-ACP-dehydratase
7	Crotonyl-ACP + NADPH + H^+	$\longrightarrow$	butyryl-ACP + $NADP^+$	Enoyl-ACP reductase

Regulation of Gene Expression

The process by which biological information contained in the gene is made available to the cell is termed gene expression.



The first stage of gene expression is called transcription wherein the coding strand of the gene acts as a template for synthesis of RNA molecules. The second stage is called translation wherein the mRNA directs synthesis of a polypeptide, the amino acid sequence of which is determined by the nucleotide sequence of the mRNA.

Gene expression can be controlled at the level of translation or at the level of transcription. Initiation of mRNA synthesis (level of transcription) is a primary control point in the regulation of gene expression. Transcriptional regulation of the gene can be controlled by regulatory proteins that bind to specific sites on DNA. These specific DNA sequences, also sometimes called regulatory elements can be scattered both upstream and downstream of the transcription start site for a gene. Figure 3 is a diagram of the transcriptional control region of a gene.

The control region in the immediate vicinity of a transcription start site is called the promoter; regions that regulate a promoter from a distance and in an orientation-independent fashion are called enhancers. Example of promoter elements are TATA and CCAAT boxes.

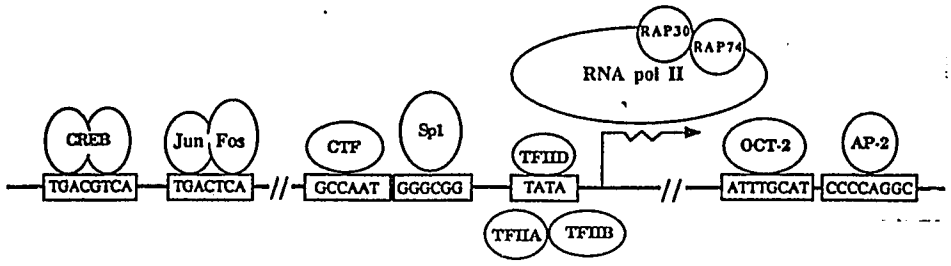


Figure 3. Features of the Transcriptional Control Region for a Mammalian Gene.

The transcription initiation site is indicated by an arrow. Boxed sequences indicate promoter and enhancer regions. The DNA binding proteins that associate at the control regions are symbolically represented and include CREB, Jun, Fos, CTF, Sp1,OCT-2, and AP-2. (Source:Mitchell, P.J., and Tjian, R. (1989). Transcriptional Regulation in Mammalian Cells by Sequence-Specific DNA Binding Proteins. Science, 245, p.372)

Control of FAS Gene

One way to regulate fat biosynthesis is to regulate the activity of FAS, the enzyme that catalyzes the reaction. Studies have shown that regulation of FAS synthesis and activity is affected by diet and hormone, (Goodridge, 1987) . The studies have shown that starvation decreases and refeeding increases the activity of avian fatty acid synthase, principally by regulating transcription of the gene.

In chick-embryo hepatocytes in culture, the stimulatory effect of feeding on fatty acid synthase activity is mimicked by adding T_3 and insulin. It has also been shown that T_3 alone stimulates transcription of FAS by 4 - 6 fold. When added alone, insulin has little or no effect on transcription,

mRNA level or enzyme activity. In combination with T_3, however, insulin amplifies the response to T_3 by about 2-fold, leading to an overall increase of about 10-fold (Stapleton, Mitchell, Salati, & Goodridge, 1990).

Triiodothyronine (T_3)

A hormone is a chemical agent which is released from one group of cells and travels, via the bloodstream, to affect one or more different groups of cells. T_3 belongs to the group of hormones that interact with their target cells by binding to intracellular receptors, with the receptor-hormone complex modulating gene expression.

The nuclear receptor for T_3 has been characterized as a chromatin-associated protein and is believed to be encoded ny the proto-oncogene c-erbA, (Sap et al., 1986; Weinberger et al., 1986). The proposed mechanism of action is that the T_3 receptor binds with high affinity to specific DNA sequences adjacent to or within T_3 responsive genes and alters their rate of transcription. Specific DNA sequences which confer T_3 regulation on a gene are called T_3 - response elements (TRE).

Figure 4 shows that TREs for different genes have limited sequence identity and are located at different sites within a gene. To date the TRE for the FAS gene have not yet been established.

Objective of the Study

Kameda and Goodridge (1991) were able to isolate and partially

characterize the promoter region of the goose fatty acid synthase gene. Since our laboratory's working model system has been chick-embryo hepatocytes in culture and assuming sequence similarity between the two avian species goose and chicken , we set to study the following objectives: (a) isolate FAS promoter region in chicken using PCR, and (b) characterize the TRE in the promoter region.

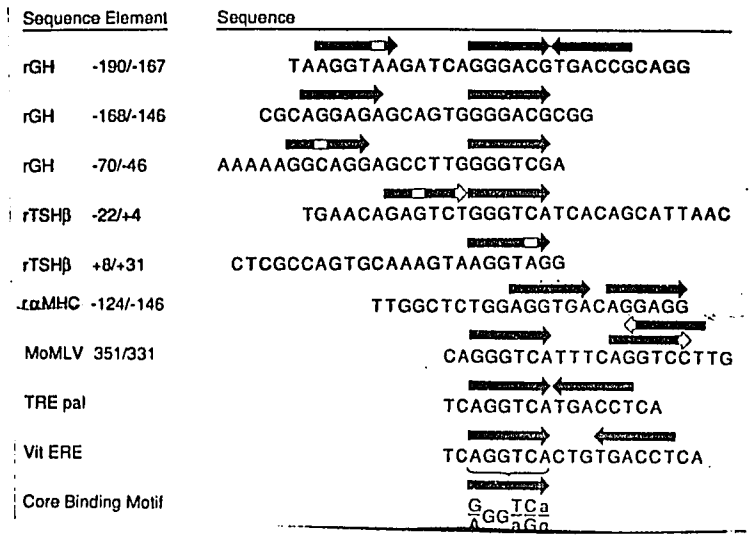


Figure 4. Established TREs for Some Genes.

Sequences illustrated are from rGH, rat growth hormone gene; rTSHß, rat thyroid stimulating hormone gene; hTSH, human TSH gene; rMHC, rat - myosin heavy chain gene; MoMLV, moloney murine leukemia virus gene; TRE pal, a palindromic variant of rat GH TRE; Vit ERE, Xenopus vitelligenin ERE. Arrows highlight sequences which correspond to a proposed TRE core binding motif and indicate the direction of this motif. Base pairs which do not match with the core binding are indicated by an open region within the arrow. (Source: Glass, K.C., and Holloway, J.M. (1990). Regulation of Gene Expression by the Thyroid Hormone Receptor. Biochimica et Biophysica Acta, 1032, p.167).

Significance of the Study

Isolation and characterization of the TRE of the FAS gene can contribute to the understanding of the mechanism on how fat biosynthesis can be regulated. Understanding the nature of the hormonal regulation of this major metabolic pathway is essential to understanding and controlling the problems of obesity, diabetes mellitus, and cardiovascular related diseases.

MATERIALS AND METHODS

PCR of the FAS Promoter and HPRT Gene

Isolation of Chicken Genomic DNA

A one month old white leghorn chicken was sacrificed by decapitation. The liver was isolated and minced with a sterile razor blade. Genomic DNA from the liver was isolated using the fresh tissue DNA preparation (Davis, Dibner, & Battey, 1986).

Primers for PCR

The published nucleotide sequence of the promoter region of the goose FAS gene is indicated in Figure 5. Based on this sequence, 20 bp oligonucleotides were chosen to serve as primers for the polymerase chain reaction (PCR). According to standard PCR protocols, efficient primers should have the following characteristics: (a) 50-60 % G + C content (b) melting or dissociation temperature between 55°C and 80°C (c) dimer formation negligible, and (d) no stretches of polypurines or polypyrimidines. The nucleotide sequence of the published promoter region of the goose FAS gene was analyzed on the oligo program primer analysis software (National Bioscience, 1990) to choose primers that will satisfy the above conditions.

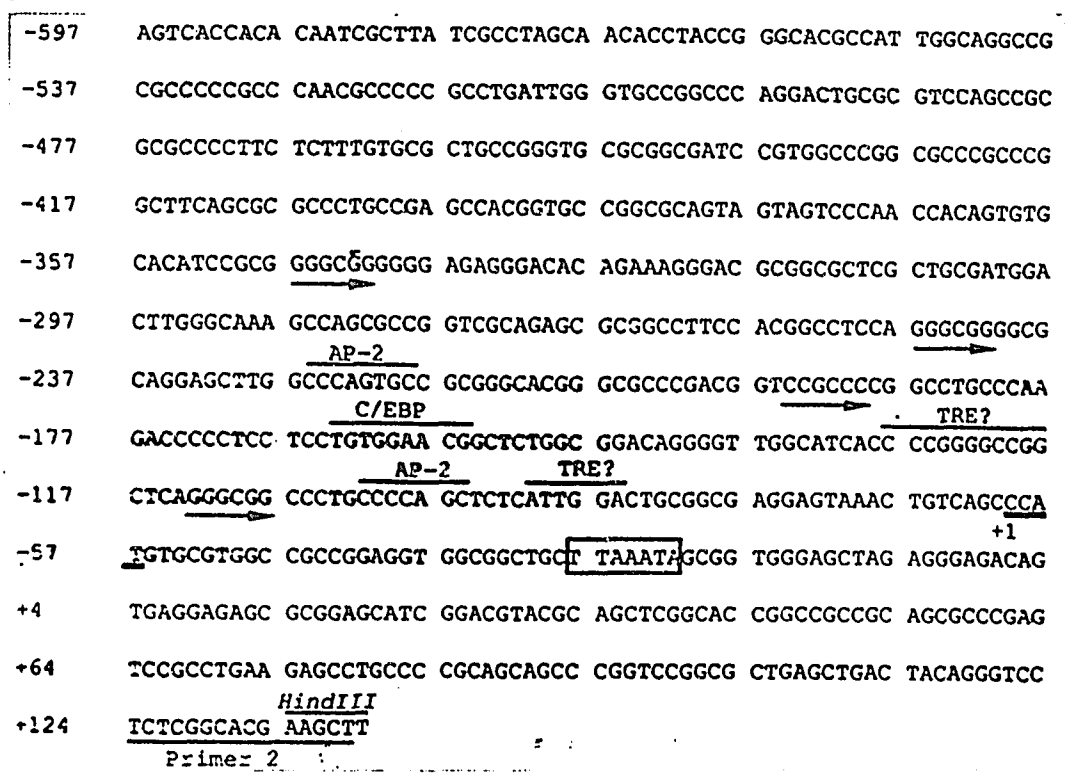


Figure 5. Nucleotide Sequence of the 5'- Flanking Promoter Region of the Goose FAS Gene.

The transcription initiation site is assigned position +1. A potential TATA box 28 bp upstream of mRNA start site is boxed. A CCAAT homology 60 bp upstream of mRNA start site is denoted by the heavy line. (Source: Kameda, K. and Goodridge, A.G. (1991). Isolation and Partial Characterization of the Gene for Goose Fatty Acid Synthase. The Journal of Biological Chemistry, 266, (1), p. 424).

PCR of the Promoter Region of Chicken FAS

In vitro amplification was carried out with a DNA thermal cycler using the Hybaid Thermal Reactor Model # HBTR1 (Hybaid Limited, U.K.). The reactions were carried out in a final volume of 100 μl containing 1 μM of

each primer, 200 μM dNTPs, 100 mM Tris HCL pH 8.3, 500 mM KCl, 30 mM $MgCl_2$, 0.5 μg chicken genomic DNA, and 2.5 units of Ampli Taq DNA polymerase (Boehringer Mannheim). Amplification conditions were 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 2 minutes, and extension at 72°C for 3 minutes. 20 μl aliquot of the amplification reaction mixture was analyzed by electrophoresis on 1.0 % agarose gel.

Optimization of PCR Reaction Conditions

$MgCl_2$ concentrations ranging from 20 mM to 50 mM were tried with all other parameters held constant.

Annealing temperature was changed from 55°C to 60°C.

Addition of 5 % dimethylsulfoxide, DMSO, to the reaction mixture was also tested.

PCR of the Exon 3 of the Chicken HPRT Gene

To serve as a control for our PCR reaction and to test the integrity of the isolated chicken genomic DNA, 1 μM of each primers designed from and used to amplify the mouse hypoxanthine phosphoribosyl transferase, HPRT, gene were employed to determine if they could also amplify the HPRT of chicken. The reaction conditions were similar to those used to amplify the promoter region of the chicken FAS gene except that 15 mM was the final $MgCl_2$ concentration in the reaction mixture.

Tests on the 1.6 EH CAT Chimeric Gene

Isolation and Purification of the 1.6 EH CAT Clone

A clone (1.6 EH CAT) containing the promoter region of goose FAS gene was obtained from A.G. Goodridge, University of Iowa. This clone was constructed by Kameda and Goodridge (1991) as follows: The putative promoter region containing 1.6 kb of 5' flanking sequence and 139 bp of untranslated sequence of the fatty acid synthase gene was inserted 5' to the bacterial chloramphenicol acetyltransferase (CAT) gene. This fragment was inserted into the Hind III--Apa I site of pBluescript KS (+) (Stratagene), a phagemid containing the origin of replication.

Large scale plasmid DNA preparation and isolation of this 1.6 EH CAT was performed for subsequent analysis (Sambrook, Fritsch, & Maniatis, 1988). The plasmid DNA was then purified using the acid phenol extraction procedure as outlined in the Promega Erase-A-Base system technical manual (Promega, 1991).

Preparation and Maintenance of Chick Embryo Hepatocytes

Eggs from white leghorn chickens purchased from Town Line Poultry Farm (Zeeland, MI) were incubated in an electric forced draft incubator at 39°C and 60 % relative humidity. 19- day old embryos were sacrificed by decapitation. Liver cells were isolated (Goodridge, 1974) and suspended in Waymouth MD705/1 media containing penicillin and streptomycin on either 35 mm or 60 mm Falcon plates. The cells were then incubated at 39°C in

an atmosphere of 95 % air, 5 % CO_2.

Transfection of 1.6 EH CAT DNA Into Chick Hepatocytes

After about 24 hours of incubation of the hepatocytes, the medium was changed to one containing a DNA/lipofectin complex solution. Basically, 6 μg of acid phenol purified 1.6 EH CAT DNA and 30 μg of lipofectin (Gibco/BRL) were diluted separately in Waymouth MD705/1 media. The two separate dilutions were then mixed and incubated at room temperature for 15 minutes to allow the complex to form. The DNA/lipofectin mix was then added to the hepatocytes and incubated for 6 hours. To one-half of the plates, the lipofectin/DNA mix was changed with Waymouth MD 705/1 only, while to the other half, the mix was changed with Waymouth MD705/1 media containing 300 ng insulin per ml and 1.5 μg T_3 per ml. The cells were then incubated at 39°C in 5-10 % CO_2 for 36--48 hours.

CAT Assay

The cells were harvested 48 hours later. Cell lysates were prepared by three cycles of freezing and thawing. Cellular debris was removed by centrifugation and the supernatant was heated at 60°C for 10 minutes to inactivate endogenous acetylase. The chloramphenicol acetyltransferase activity was determined in the supernatant solution using the thin layer chromatography method as outlined in the Promega Technical Bulletin for its CAT Enzyme Assay System (Promega, 1989).

Preparation of Deletion Constructs

Deletions of the 1.6 EH CAT plasmid were carried out according to the Promega Erase-a- Base System technical manual (Promega, 1991). Nicked and linear DNA were selectively removed from the 1.6 EH CAT DNA by acid phenol extraction. About 5 μg of the purified plasmid DNA was restriction digested with Sac I and Bam HI. Sac I generates the exonuclease III resistant 3' overhang. Exonuclease III deletion of the 1.6 EH CAT was then carried out at 23.5°C (room temperature) every 2 minutes. Using this condition, around 200 bp are deleted for each time point. The deletions were then ligated and transformed in high efficiency JM 109 competent cells.

RESULTS AND DISCUSSION

Quantitation of the Isolated Chicken Genomic DNA

The isolated genomic DNA from the chicken fresh liver tissue was measured for the following parameters as illustrated in Table 2:

Table 2

Parameters Measured for the Isolated Chicken Genomic DNA

A_{260}	=	0.0678	DNA concn.	=	0.678 $\mu g/\mu l$
A_{280}	=	0.0363	DNA purity	=	1.87

PCR of the FAS Promoter From the Chicken Genomic DNA

The nucleotide sequence of the published promoter region of the goose FAS gene when checked on an oligo program primer analysis software (National Bioscience, 1990) did not give oligonucleotides that satisfy all of the criteria for efficient primers and at the same time span the entire promoter region. We ended up with the following primers (Table 3) that satisfied most of the criteria.

Table 3

Primers Used for PCR

primer	sequence	G+C	T_d
FAS 06	5' CCACACAATCGCTTATCGCC 3'	55 %	68.4°C
FAS 658	5' CAGGCTCTTCAGGCGGACTC 3'	65 %	69.2°C

Using the above primers which were based on the published sequence of the goose promoter, we expected to obtain a 671 bp amplified fragment. After PCR, multiple bands were obtained, however, a fragment between markers 615 bp and 738 bp also appeared. This fragment was assumed to be the one of interest (Figure 6).

To obtain maximal amplification of the expected size fragment, different PCR conditions were tested.

Magnesium Ion Concentration

Optimization of the free magnesium ion concentration in PCR reactions can result in an increase in primer specificity. Four different $MgCl_2$ concentrations (20, 30, 40, and 50 mM) were studied combined with the other parameters held constant. Figure 7 presents the results obtained using different $MgCl_2$ concentrations.

Our system showed amplification of the expected fragment only at 30 mM $MgCl_2$. A lower or higher $MgCl_2$ concentration than 30 mM did not

give the expected 671 bp fragment. 30 mM $MgCl_2$ was then chosen as the condition for further study.

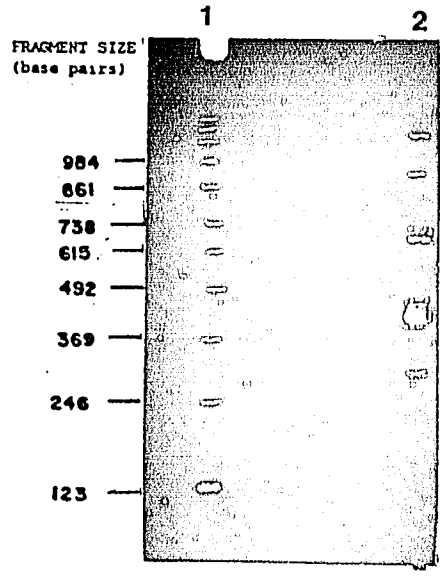


Figure 6. PCR Products From Chicken Genomic DNA.

The PCR protocol used is described in the Materials and Methods. Lane 1, 123 bp DNA ladder (BRL); lane 2, PCR fragments from chick genomic DNA.

Annealing Temperature

When total genomic DNA is used as template, a higher annealing temperature is recommended because there is a much greater chance that the primers will anneal non-specifically to the template. Theoretically raising the annealing temperature can greatly reduce false priming events because at elevated temperature only primers that are nearly 100 % complementary to template will anneal, generating a highly specific

product. A higher annealing temperature, from 55 °C to 60 °C, was tested. Figure 8 shows the PCR result using a higher annealing temperature.

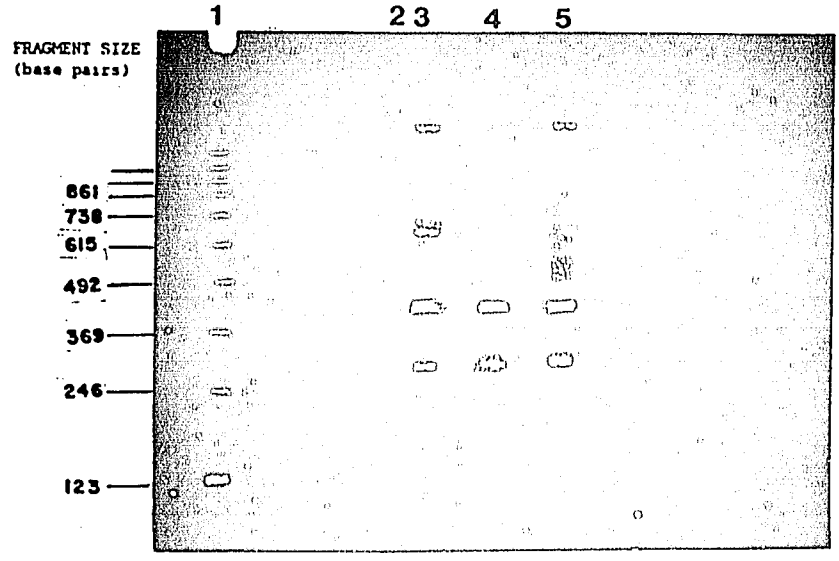


Figure 7. PCR Fragments Obtained by Varying the $MgCl_2$ Concentration.

Lane 1, 123 bp DNA ladder (BRL); lane 2, PCR in 20 mM $MgCl_2$ buffer; lane 3, PCR in 30 mM $MgCl_2$ buffer; lane 4, PCR in 40 mM $MgCl_2$ buffer; lane 5, PCR in 50 mM $MgCl_2$ buffer.

With our system, increasing the annealing temperature, gave us more amplified fragments. Therefore, the 55 °C annealing temperature was selected.

Use of DMSO

The use of dimethyl sulfoxide (DMSO) has been reported to improve the DNA amplification. The mechanism behind the action of DMSO is

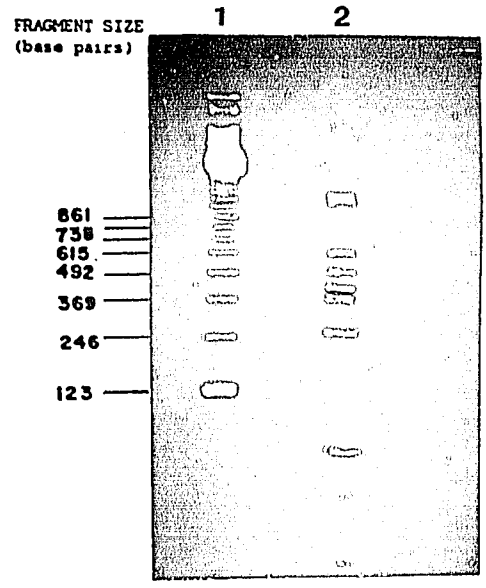


Figure 8. PCR Fragments Obtained at 60 °C Annealing Temperature. Lane 1, 123 bp DNA marker (BRL); lane 2, PCR at 60 °C annealing temperature.

unknown, but it has been suggested that enhancement of PCR by this compound is associated with the general property of organic solvents to destabilize DNA in solution (Lee, Mizusawa, & Kakefuda, 1981). Organic solvents cause dehydration in the microenvironment of the DNA leading to structural perturbation or increased unwinding of the double strand DNA. With our system addition of 5 μl DMSO did not enhance the specificity of our PCR product (Figure 9).

Unfortunately after evaluating different parameters for the PCR reaction, a single band of the expected fragment was not obtained. Isolation of the PCR fragment by tombstoning and subsequent cloning of this fragment was also attempted but was unsuccessful.

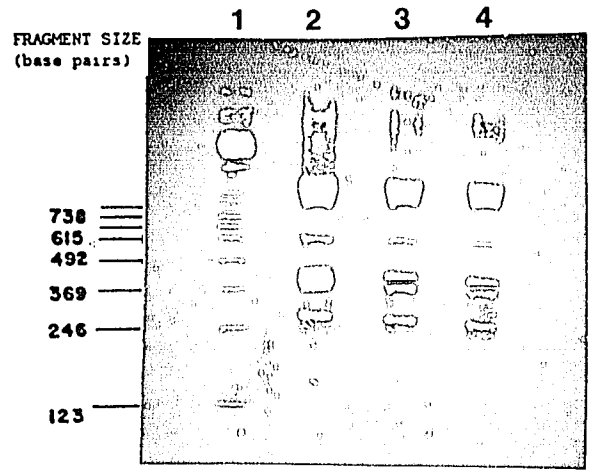


Figure 9. PCR Fragments Obtained With DMSO Addition.

Lane 1, 123 bp DNA ladder (BRL); lane 2, PCR without DMSO; lanes 3-4, PCR with 5% DMSO.

Ligation of PCR Fragments to a Vector

The next method for cloning the fragment of interest utilized the shotgun approach. The entire PCR reaction mix was ligated into the plasmid vector pCR 1000 (InVitrogen), hoping that the fragment of interest, among others, would also get ligated.

Transformation and Restriction Analysis

The ligation mix was then transformed into INV F ' bacterial cells. The vector contains the ß galactosidase gene for blue and white color selection of colonies. A number of white positive colonies which contain the PCR inserts were selected and subjected to DNA mini-preps followed by restriction analysis. Figure 10 shows the results of the restriction analysis.

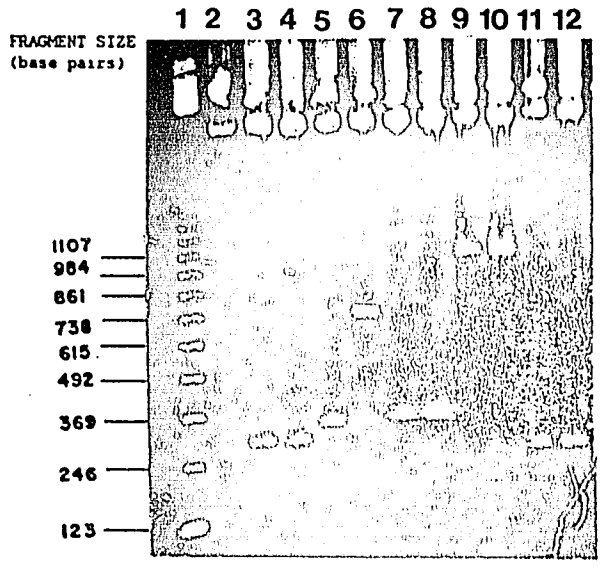


Figure 10. Restriction Analysis Fragments From Cloned Chicken FAS Putative Promoter Gene.

Lane 1, 123 bp DNA ladder (BRL); lane 6, fragment of interest located between markers 615 bp and 738 bp.

The result observed in lane 6 of Figure 10 indicates that this plasmid contained an insert size between 615 bp to 738 bp which is similar in size to the fragment of interest.

DNA Sequence of the Fragment of Interest

The partial DNA sequence of the 671 bp fragment of chicken genomic DNA is shown in Figure 11 . TATA and CCAAT boxes are present which are indicative of a promoter region.

However, when this sequence was compared to the published nucleotide sequence of the 5 ' flanking region of the goose FAS gene, no

significant homology was obtained.

CCGAGGTGAG AAGGGTTCCA CACAATCGCT TACTGCCCCA TTCTATCACA

TCTTCATTTT AGAGCAGACC ATCAAGTCAG GACAGGAGCT ACCAAAACAC

ATAGGGCTAA GCTCAGCTTT ATCAAGGGAT TGTTGTCAGT ATCTAAAACA

AAAAGGATAT TCACCCTTTA TGCCCTTATA TAGCTCCGTC TATCCTGATA

CCACTACCAA TGGGGATTTT CAGATGCTTT GAAGGTAGGC TGCTCCCTGC

TATACACACC TACAAATACT CGCTCTCTCC TCCTCCAGGC TAATTGCAGC

ATTCATCTGA CCAGTGTCTG TGTACTATGA AGTGAAGTGA AACCACTGGG

CCCTGAAGGC CAATCCAACC CCAGAAACCA ACTTCGCATT GTGCCCGGCC

AGAGTCCGCC TGAAGAGCCT GAAACGGTTC

Figure 11. Partial Nucleotide Sequence of 671 bp PCR Fragment From Chicken Genomic DNA.

A potential TATA sequence is boxed. A CCAAT homology is denoted by a heavy underline.

At this point, a question remained concerning whether or not goose and chicken DNA would be as similar as expected or perhaps the primers were not as selective as possible. Another primer, (FAS 255 : 5 ' GGGGGGAGAGGGACACAGAA 3 ') was ordered and tested.

According to the sequence information from the goose FAS promoter, the primers FAS 255 and FAS 658 should yield a 423 bp fragment. Figure 12, lanes 2 & 3, shows the products obtained when chicken genomic DNA was amplified with this new set of primers. A fragment of approximately 450 bp was obtained along with other fragments of various sizes.

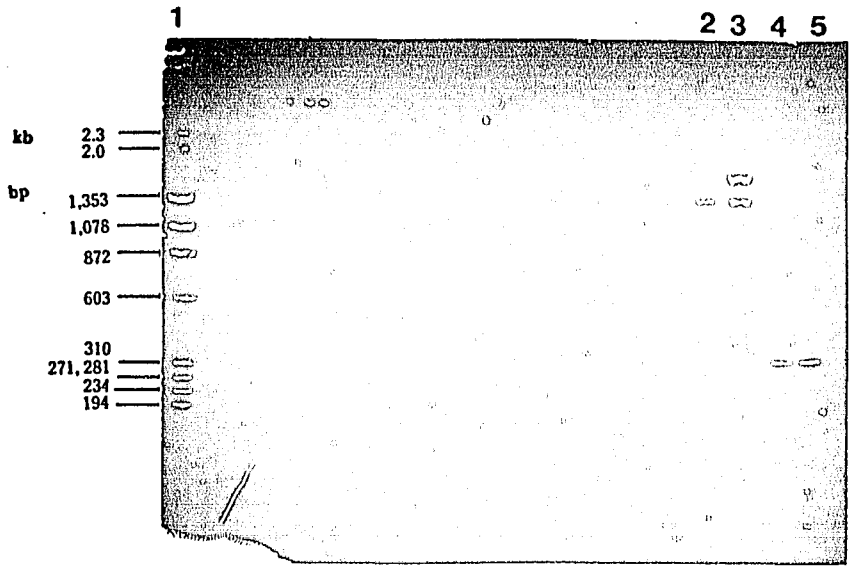


Figure 12. PCR Products Obtained Using Primers FAS 255 and FAS 658.

Lane 1, HIND III-cut DNA and Hae III -cut øX174 RF DNA markers; lanes 2 &3, PCR fragments from chicken genomic DNA; lanes 4 & 5, PCR fragments from 1.6 EH CAT plasmid.

The cloned goose genomic fragment containing the putative promoter linked to the CAT gene (1.6 EH CAT) was obtained from Goodridge, University of Iowa, and used to test the specificity of the primers.

Using primers FAS 255 and FAS 658, a PCR fragment was obtained from the 1.6 EH CAT construct. Only one fragment was obtained, however, the size was not as expected (Figure 12, lanes 4 & 5).

PCR of the HPRT Gene From Chicken Genomic DNA

Coincident with the above experiments, the integrity and amplifyability of the chicken genomic DNA was also evaluated by using primers

available (L.C. Ginsberg Laboratory) for exon 3 of the mouse HPRT gene. The HPRT primers used were: REX 3 5 ' TCAGACTGAAGAGCTACTGT 3 ' and LEX 3 5 ' GACTGAAAGACTTGCTCGAG 3 '. Using similar protocols for amplification as described, one band was amplified and it corresponded to the appropriate size (Figure 13).

The DNA fragment was ligated to pCR 1000 plasmid vector and transformed into INV F ' bacteria (InVitrogen, 1990). The DNA inserts obtained after mini prep and restriction analysis of the various colonies with EcoR I and Hind III are shown in Figure 14. A search in the Gen Bank™/EMBL DNA sequence data bank gave no information available on chicken HPRT gene. Therefore, the DNA insert was sequenced using the Sanger method (Figure 15).

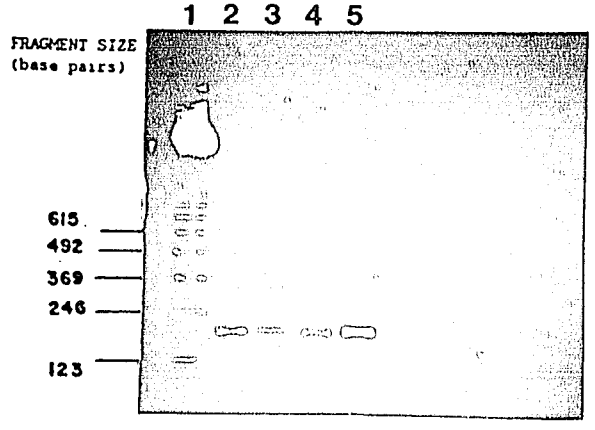


Figure 13. PCR Products of Mouse and Chicken HPRT Gene.

Lane 1, 123 bp DNA ladder (BRL); lane 2, PCR product of mouse HPRT; lanes 3-5, PCR product of chicken HPRT.

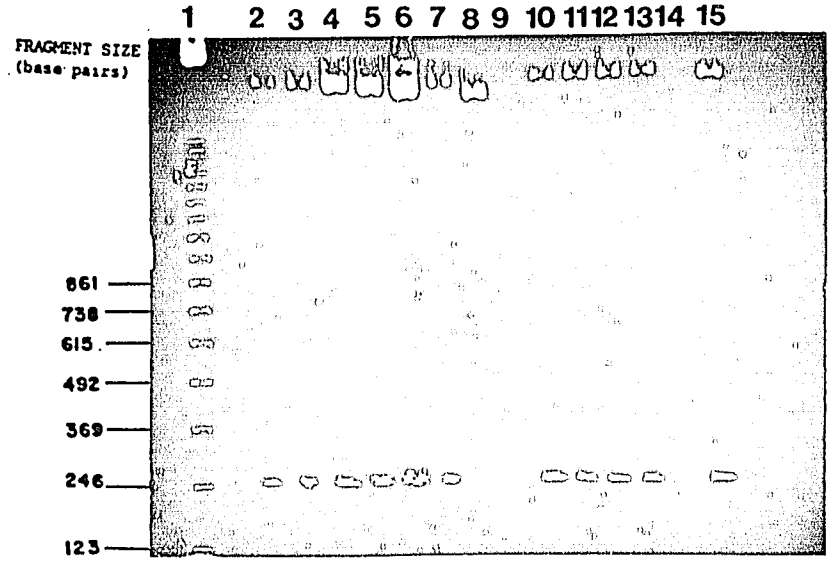


Figure 14. Restriction Analysis Fragments From Cloned Chicken HPRT Gene.

As illustrated in Figure 15, there is a very high sequence homology between the exon 3 of the mouse HPRT gene and the chicken HPRT gene. One base pair change is observed and the chicken DNA appears to have 3 bases deleted.

Based on the above results, the chicken genomic DNA that has been used is easily amplified. However, we still cannot explain why multiple bands are obtained in our PCR when using primers based on the published nucleotide sequence of the FAS gene, nor can we currently explain why the fragment size of chicken and goose products differ or why the goose product appears shorter than expected.

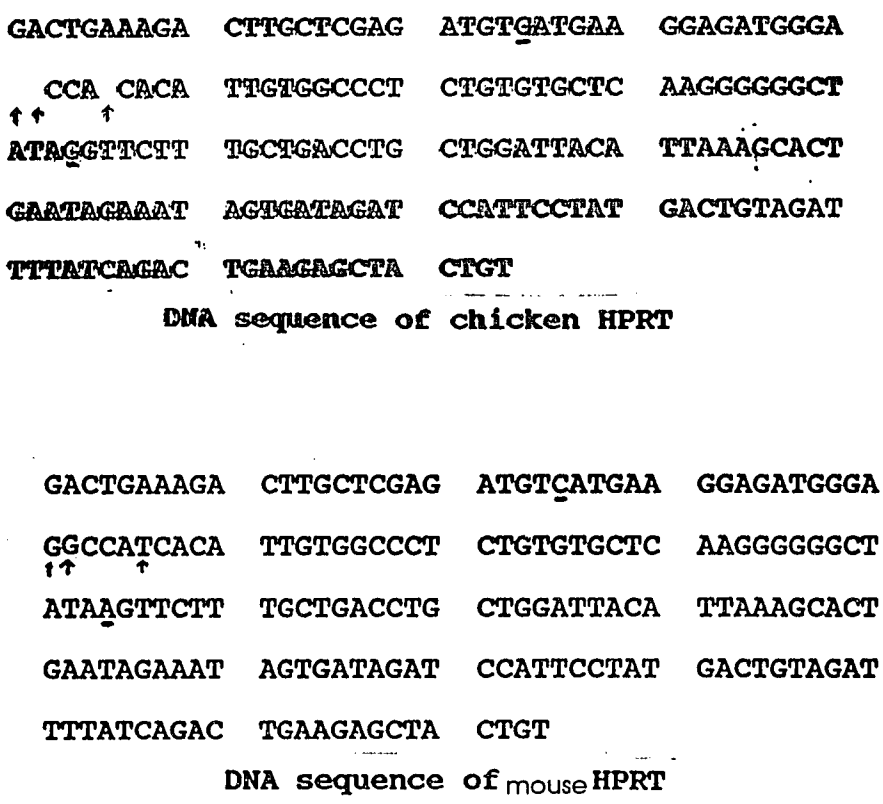


Figure 15. Comparison of the Nucleotide Sequence of the Exon 3 of Mouse and Chicken HPRT Genes.

Arrows indicate the nucleotides that are missing in the chicken HPRT gene but are present in the mouse HPRT gene. The base pair change is denoted with a heavy line.

Test for CAT Activity of the 1.6 EH CAT Gene

As published, the 1.6 EH CAT chimeric gene contains part of the FAS promoter region, and this promoter is responsive to T_3.

The construct was tested for expression and responsiveness in chick-embryo hepatocytes in culture. Figure 16 shows the result of this test.

Indeed, in our hands, the 1.6 EH CAT chimera induces CAT activity and the hormones insulin and T_3 enhance this stimulation.

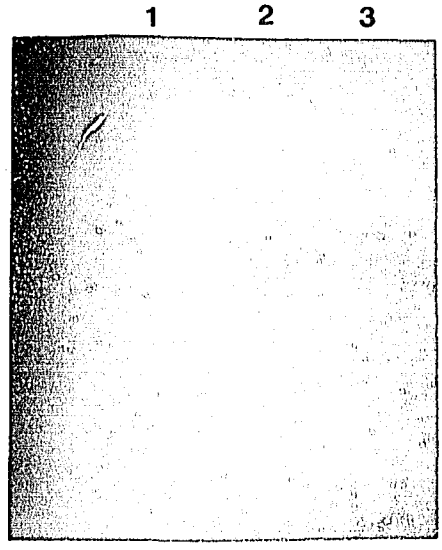


Figure 16. CAT Activity Induced by the 1.6 EH CAT Cimeric Gene.

Lane 1, CAT activity induced by standard chloramphenicol acetyl transferase; lane 2, CAT activity induced by 1.6 EH CAT gene with no hormones present; lane 3, CAT activity induced by 1.6 EH CAT gene with Insulin and T_3 added.

Deletions on the 1.6 EH CAT Gene

To localize which part of the promoter region of the 1.6 kb fragment is responsible for the triiodothyronine response, several deletions were made using exonuclease III digestion. Exonuclease III specifically digests DNA from a 5 ' protruding end while leaving a 4 base 3 ' protruding end intact.

Based on the map of pBluescript KS (+) Stratagene vector (Figure 17) and the location of the 1.6 EH CAT insert, we chose Sac I

endonuclease to generate the exonuclease III resistant 3 ' overhang and BamH I to generate the 5 ' overhang sensitive to exonuclease III digestion.

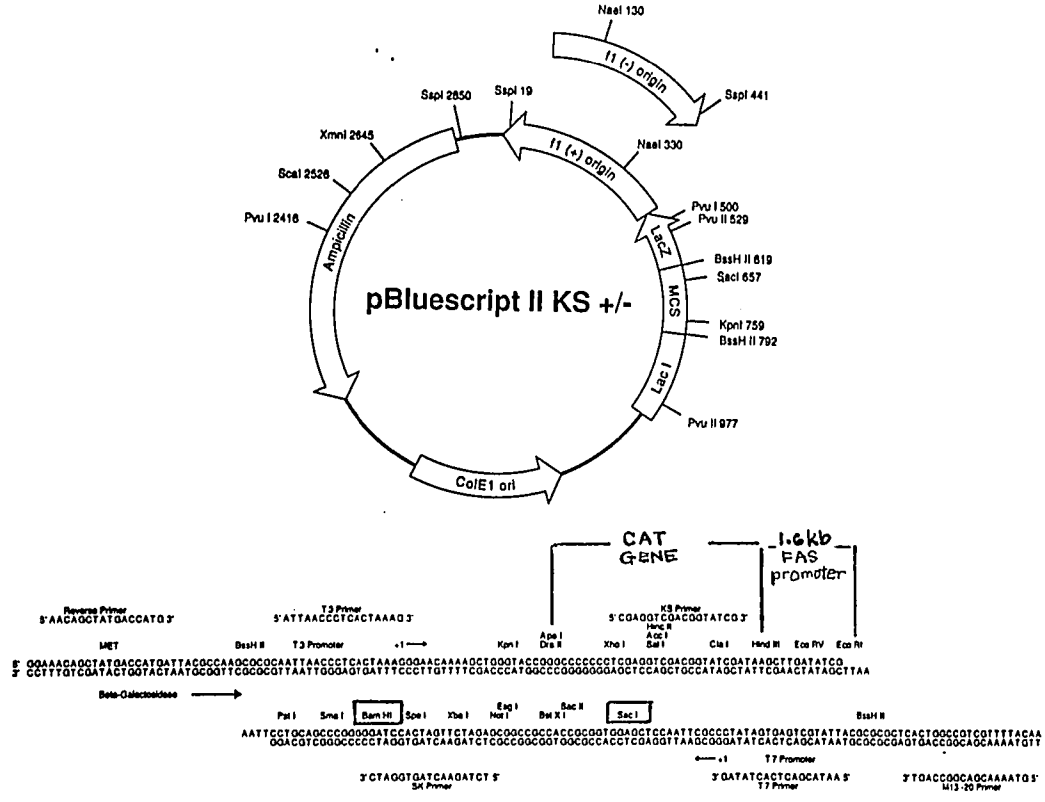


Figure 17. Map of pBluescript II KS +/- (Stratagene) Vector.

The CAT gene is inserted in the Apa I-Hind III sites. 1.6 kb of the FAS promoter is located in the EcoR I- Hind III sites 5 ' to the CAT gene. Sac I and Bam HI, the restriction enzymes used to generate the correct ends for exonuclease III digestion are boxed.

Figure 18 shows that Sac I and Bam HI restriction enzymes cut only at unique sites within the polylinker region of pBluescript KS +/- vector and they do not cleave the 1.6 kb EH CAT insert.

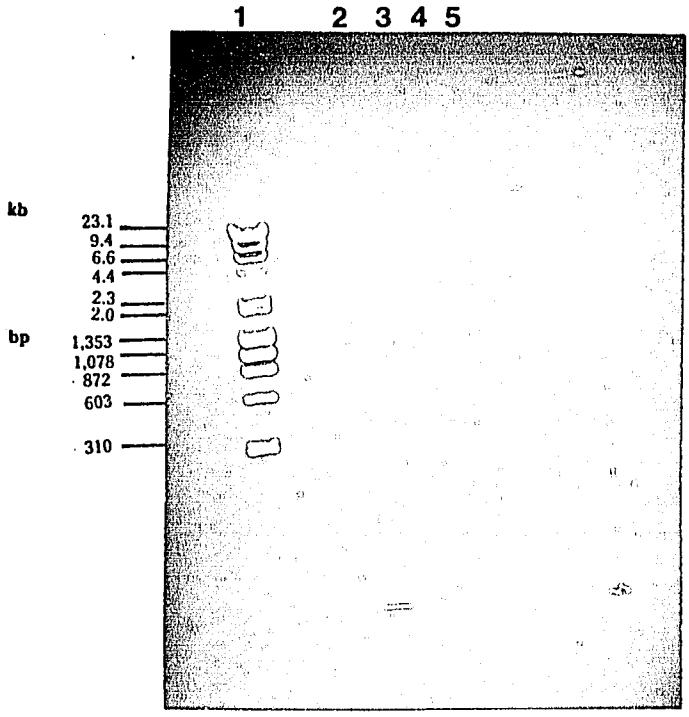


Figure 18. Restriction Analysis of 1.6 EH CAT Gene.

The acid phenol purified 1.6 EH CAT gene was restriction digested for 2 hours with Bam HI to generate the exonuclease III sensitive 5 ' overhang followed by 4 hours incubation with Sac I to generate the exonuclease III resistant 3 ' overhang.

Before restriction digestion was performed, the 1.6 EH CAT plasmid was purified from linear and nicked DNA using acid phenol extraction. It takes about 5 - 6 days of emulsification and stabilization with 50 mM TE buffer, pH 4 for the phenol to reach pH 4.03. The pH of the phenol is critical. pH 4.1 will not be able to remove the nicked molecules.

Figure 19 shows the deletions obtained after exonuclease III digestion of the restricted 1.6 EH CAT gene. The presence of secondary bands are also observed at each deletion time point.

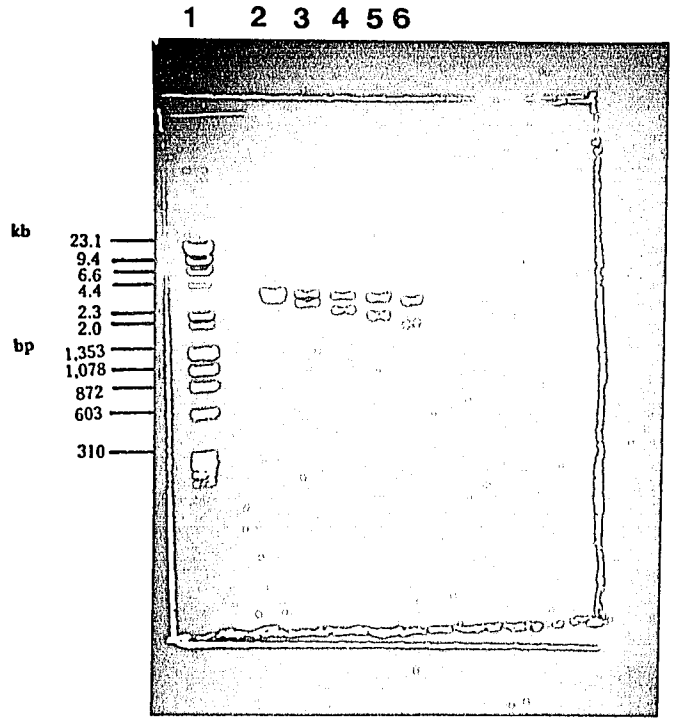


Figure 19. Deletants Obtained After Exonuclease III Digestion of the 1.6 EH CAT Chimeric Gene.

The deletion conditions using exonuclease III are described under Materials and Methods. Lane 1, Hind III-cut dna and Hae III-cut øX 174 RF dna markers; lanes 2-6, 1.6 EH CAT chimeric gene with 200 bp deleted per deletion time point done successively.

According to the troubleshooting procedure in the protocol (Promega, 1991), the secondary bands appear when the protecting restriction enzyme, in this case Sac I, failed to cut completely. The singly cut molecules would be digested at twice the rate of those that were double cut so a second, smaller specie would be present at each deletion time point. The recommendation is to repeat the initial digestion using more enzyme for a greater time period. More Sac I was used and restriction digestion was

carried for 6 hours, but still the secondary bands appeared.

Though exonuclease III digestion of the restriction digested DNA gave secondary bands we still proceeded to circularize each deletion-containing vectors by ligation. The ligation mixtures per deletion time point were then transformed into JM 109 E. coli strain competent cells. Two types of colonies per deletion time point would be expected since the DNA ligated contained a secondary band. Therefore, one result would show the insert as the primary band and the other the secondary band.

A number of clones derived from each deletion time point have been isolated, stored as glycerol stocks, and will be subjected for future study.

CONCLUSION AND RECOMMENDATION

In this study we were able to: (a) isolate a putative FAS promoter region from chicken liver using known goose FAS promoter sequence and the polymerase chain reaction; (b) isolate, clone, and sequence the exon 3 of the chicken HPRT gene, as a control from the experiments conducted in a; and (c) create deletions of a cloned 1.6 kb promoter region of goose liver FAS to determine the location of the T_3 response element present.

The following are recommended for further study:

1. Since the fragment obtained from the PCR of the 1.6 EH CAT using primers FAS 255 and FAS 658 does not give the expected bp, sequencing of the clone containing this fragment and also of the 1.6 EH CAT chimeric gene will counter check the published nucleotide sequence of the promoter region for goose FAS gene.

2. Construct a chimeric DNA containing the amplified chicken sequence ligated to the CAT gene to test for putative promoter activity.

3. The identity of the plasmids from the deletion clones should be verified by dideoxy sequencing.

4. To functionally map the regions of the 5 ' flanking sequence of goose FAS promoter region that are important in mediating cell-specific and regulated expression by T_3 , CAT activity assay on each of the verified

deletion constructs should be performed.

5. Once the T_3 responsive deletion construct is identified, then, gel shift assay and DNA footprinting should be done to fully delineate the TREs.

6. If none of the deletion constructs give any CAT activity, then most probably the T_3 responsive elements are located outside the 1.6 kb fragment. In that case, additional 5 ' flanking sequences (from cos FAS 4-- a clone containing over 30 kb of 5 ' sequence and which was also sent by A.G. Goodridge) can be cloned into a CAT expression vector and tested for its ability to induce CAT activity in the presence of thyroid hormone.

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