http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences, 2 (4): 1236-1239, 1999 Research Article

The Cloning and Sequencing of the Regulatory Element of Goat Beta-Casein Gene

Omar Shair*
Beijing Agricultural University, P.O. Box. 100094, P.R. China
*Present Address: King Abdulaziz City for Science and Technology,
Institute of Natural Resources and Environmental Research,
P.B. Box. 6086. Riyadh 11442 Saudi Arabia

Abstract
So far, many promoters have been isolated and used to drive the expression of foreign gene in the mammary gland. However, none of these promoters were specific and the expression in the mammary gland tissue was associated with expression in other tissues. The need for discriminating sequences, which can drive the expression of foreign gene specifically in the mammary gland, is obvious. This study is intended to clone specific regulatory elements of 11 casein gene, which may drive the expression of every gene specifically in the mammary gland. The isolation and purification procedure of the genome goat from the mammary gland and the isolation and purification of Lambda vector was performed.
The right and left arms were of the phage were also purified and packaged with packagene. A goat genome library consisting of 2. 1 X 10^5 phages was constructed and screened by using in situ hybridization technique. Following the procedure of Sambrook *et al.* (1989) we performed the *in-situ* hybridization technique. The probe was prepared through Polymerase chain reaction (PCR) using the goat genome as template. The probe was used in the *in-situ* hybridization technique to screen the library after cloning it into puc 18 vector. Sall and BamHI were used for the analysis of the recombinant phage. The sal 1 fragments were cloned in pGEM5z vector. The partial sequencing of the 4.5kb long Sal 1 fragment cloned in pGEMbz was performed following the procedure of Sambrook *et al.* (1989). In this study we suggest that the goat 11 casein promoter may be very strong and tissue specific promoter, which may contribute in the progress and development of the mammary gland expression for the production of valuable proteins in the milk.

Introduction

The easy automation of DNA sequencing has greatly facilitated in the characterization of mammary cDNA and genes associated with milk proteins in various species. Some 60 cDNA and 20 genes from 12 species have already been completely sequenced (Bonsing *et al*., 1988). These cDNA or genes can now be modified *in vitro* by site directed mutagenesis and then expressed in various systems, such as bacteria, yeast, Bculovirus infected cells and Cos cells after insertion into adequate vectors. Studies of genetic polymorphism at the nucleotide level have led to the discovery of new alleles (Roberts *et al*., 1992). Expression analysis of native or modified genes using *in vitro* transcription systems, mammary cell lines, and transgenic animals has greatly improved the knowledge of the functioning of milk protein and has offered the potential for the genetic modification of milk composition (Persuy, 1992).
Over the last years, using biotechnology, researchers developed mice, which demonstrated promoter elements with tissue specificity driving on hormonally regulated fusion DNA to express products, (Reddy, 1991) however, only a limited number of promoter elements have been introduced in to domestic farm animals.

Materials and Methods

A high molecular weight DNA was isolated from lactating goat mammary gland by using methods described by Sambrook *et al.,* 1989. It was not an easy task to get very pure goat genome DNA and the required length of the fragment. By using phenol and ether, highly pure Genome was extracted, which was between 23 and 19kb long. First Enzymatic Digestion Pilot Experiment was performed to establish the exact ratio of enzymes to DNA required to fully digest the DNA then Sau3 was used to digest the DNA. In order to isolate the required length of fragments from the rest, the Sucrose Gradient Centrifugation technique was applied (Sambrook *et al.,* 1989) and analyzed by electrophoresis by using λ HindIII Marker as indicative of size.
All the tubes which contained fragments 23<19kb long were combined and used for further construction of the Genome library. Before constructing the library it was also necessary to purify the fragments because recovered fragments by Sucrose Gradient centrifugation contained sucrose. Elutiploid and 2X low salt and high salt buffer were used to purify the fragments. After the elution was completed the DNA fragments were precipitated with 2 volume of ethanol and recovered by centrifugation at 12000 g for 10 minutes and dissolved in 50 µl TE pH 8.00 and were stored in -20°C. At this stage purified fragments were ready to be legated into a vector λ arm.
The bacteriophage EMBL3 was the phage used to prepare pure λ DNA. The EMBL3 phage was selected because it can accommodate large fragment of genome DNA, up to 20 Kb, and because this vector is particularly useful for cloning sau3 partial digests (Sambrook *et al.,* 1989) and also because *Bam*HI sites in the vector are flanked by *Eco* RI and Sall sites and, therefore, it was possible to release the cloned fragment by digesting the recombinants with Sail and EcoRI. It was not ideal to clone the fragment into the plasmid vector as the fragment was 23 Kb <19Kb, therefore, the plasmid vectors could not accommodate it. To get a vector, which can accommodate such large fragments, isolation and purification of λ DNA from EMBL3 phage was performed following the method of Sambrook *et al.* (1989) and digested with *Bam* HI and the results of the

digestion mixture, were analyzed through electrophoresis. The Right and left arms were recovered from the gel and purified through phenol and chloroform extraction. (Sambrook *et al.,* 1989). When the arms were ready they were legated to the genome fragments in order to achieve a probe specific to a promoter of β-casein gene, primer one and primer two were designed (Roberts *et al.*, 1992) in the up stream from TATA Box. Primer I consisted of 20 nucleotides (ATG, CTC.CCC, AGA, ATT, TCT, GO) and primer II 20 nucleotides (TAT.ATA, CGA, TAT, TCA, ATG, AC) also. Polymerase chain reaction products were recovered and through λ marker the length of the product was investigated as size indicative marker. The PCR product was not enough to be used directly as probe therefore, it was needed to amplify the probe in PUC 18-plasmid vector. The 135bp long was amplified in plasmid vector and then the vector was linearized by digesting it with single enzyme without releasing the 135bp long probe and directly used to screen the goat genome library constructed, PCR was used for screening the library of the goat genome DNA, (Sambrook *et al.,* 1989) so that the unneeded recombinant phage was reduced.

The 4.5 kb long ·5-casein promoter was cloned into plasmid vector and amplified. (Sambrook *et al.,* 1989). The template DNA for sequencing reaction was purified by standard method. The DNA used in the sequencing reaction, as template was not single strand, rather double stranded and was denatured by alkali in an annealing mixture consisting of annealing buffer, and forward and reverse primers. Then the annealed reaction was labeled using T7 DNA polymerizes labeling buffer and a ^{32}PdATP. 135bp nucleotides from 5' to 3' and 110 bp nucleotides from 3' to 5' flanking of the 4.5 kb long promoter were sequenced.

Results

High molecular weight of goat genome has been isolated from goat mammary gland. For the results of the electrophoresis (Fig. 1) the Goat Genome DNA isolated from Goat Mammary Gland is highly pure. The first lane on the left is lambda HindIII. The goat genome was digested partially with sau3A. After sucrose gradient centrifugation at 22,000 rpm for 22 hours at 20°C, the desired size was pooled for construction of the genome library in bacteriophage λ EMBL3. The partial digests of the goat genome fragments analyzed through electrophoresis (Fig. 2). Goat Genome fragments were of different size due to Sau3A partial digest. The first lane on the left is lambda HindIII. Polymerase chain reaction products with very small molecular weight on the right (Fig. 3). On the left is the Goat Genome used as control and in the middle is lambda HindIII marker. The PCR fragment was cloned into Puc18 vector (Fig. 4) which was used as probe to screen the library. The λ DNA was isolate from EMBL3 Phage (Fig. 5), highly concentrated lambda DNA isolated from EmbL3 phage, and digested with BamHI. The left and right arms (Fig. 6), in the middle is the staffer of the phage lane and fifth from right to the left is HindIII marker. The arms were isolated from the gel, ligated to Sau3A fragments and packaged. The Genome Library was then constructed and screened by *in-situ* hybridization. The appearance of black dots indicates recombinant phage colonies (Fig. 7). The colonies harboring the recombinant phage are big and separate and their black colour is very prominent. Sa generated fragments of the recombinant phage were clone in pGEM5Z and were sequenced (Fig. 8). The 4.5 kb fragment were cloned into pGEM5z.The second lane from left is lambda HindIII marker .The first lane from the left is pGEMSz harboring the 4.5kb fragment. The result of the two directional sequence of the gene shows partial sequence of the 4.5kb of the Goat beta casein Regulator) Elements (Fig. 9).

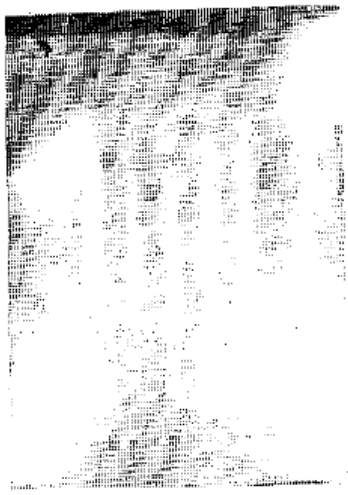

Fig. 1: High molecular weight Genome Goat DNA.
1- From left A HindIII Marker.
2-7 Genome Goat DNA

Fig. 2: Sau3A fragments of the goat Genome.
1-From left A Hind III marker.
2-13 Goat Genome fragments.

A goat genome library was constructed in the EM vector using the DNA fragments of 23<19kb long. Approximately a genome library consisting 2.1X10^5 were screened. Five positive clones were obtained and analyzed by restriction enzymes and polymerase reaction (Sambrook *et al.,* 1989). The DNA was sequence and sequence data were analyzed by reading the results

Fig. 3: Results of PCR using the goat Genome as template.
1- From left the Goat Genome as control
2- From left λ HindIII marker.
3- From left PCR product.

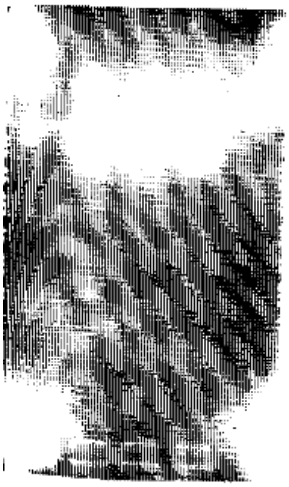

Fig. 4: PCR fragment cloned in Puc18

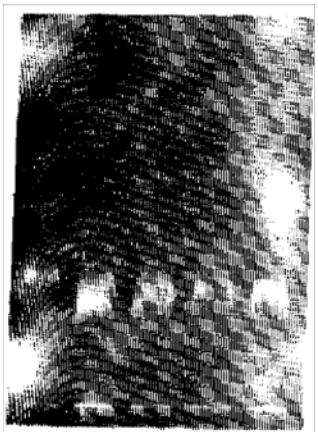

Fig. 5: A DNA isolated from EmbL3 phage

Fig. 6: Lanes 1-4 from right cntain, *Bam*HI fragments. Each lane ontains three fragments one of which is in the middle, the stuffer and the other tow fragments are the arms of the phage. The fifth lane from right contains lambDA HindIII marker

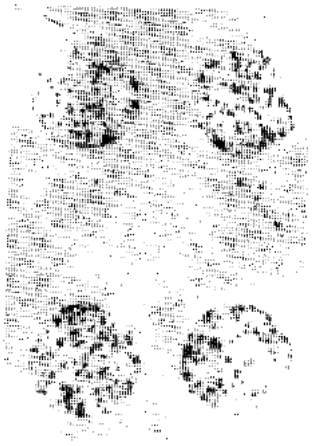

Fig. 7: Results of Hybridization, the black dots indicate recombinant phage colonies

the autoradiograph film (Sambrook et al., 1989). The target DNA to be sequenced was 4.5kb long. 135bp from 5' to 3' flanking region of the 4.5kb DNA fragment inserted into plasmid vector was sequenced and 110bp from 3' to 5' flanking was sequenced. Due to the importance of this promoter it is suggested that a further research using this promoter to drive foreign gene in the culture of mammary gland epithelial cells and finally in the mammary gland of a goat or other domestic animals.

Discussion

Isolation of high molecular weight goat genome from goat mammary gland was performed following procedure described by Ebert *et al*. (1989). Vector phage consisting left and Right arms of Lambda EmBL3 phage were purified and packaged with packagene. Goat Genome Library consisting

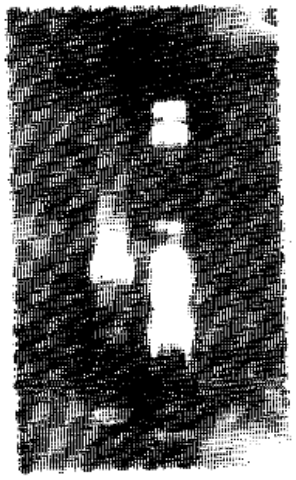

Fig. 8: 1- From right HindIII marker
2- From right the cloned Gene into pGEMS 5_z

Fig. 9: Results of sequence of the isolated regulatory elements of β-casein Gene.

$2.1x10^5$ was screened by using *in-situ* hybridization and polimerase chain reaction (PCR). Fragment of 4.5kb was cloned in PGEMZ5 and was partially sequenced. Expression of active transgene prodcuts extraneous to the target tissue may seriously affect the physiology of the animal (Behringer *et al.,* 1989). Therefore, more restrictive promoter elements are necessary to be cloned from milk protein genes. The 4.5kb long fragment of milk protein gene regulatory element isolated may restrictively drive the expression of any gene in the mammary gland.

Some discrepancies occurred with very low expression it transgenic mice for rat β-Casein transgen e comprising a $3.5kb^5$ flanking region and $3kb^3$ flanking region (Lee *et al*., 1988), In contrast, expression was high by stage specific an mammary tissue specific in transgenic mice carrying Cprine Beta-Casein transgene with $3kb^5$ flanking region a $6kb^3$ flanking region (Persuy, 1992).

Transgenic experiments have demostrated that why protein genes possess more compact promoter and gi better expression than the casein with the exception goat Beta-Casein (Persuy, 1992).

The findings of this study are in accordance with the findings of Roberts *et al*. (1992). Using these Regulatory Elements to drive the expression of foreign gene in the mamm gland, unlike other regulialory elements may specific expression only in the mammary gland of large and small domestic animals.

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