ESTABLISHING A LINK BETWEEN THE PORCINE IGF GENE AND HIGH FECUNDITY USING RESTRICTION FRAGMENT LENTGH POLYMORPHISM ANALYSIS ON AMERICAN PIG DNA VS. CHINESE PIG DNA

UNIVERSITY HONORS INDEPENDENT STUDY 499

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AUGUST 5, 1993

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Date: 9/13/92

INTRODUCTION:

Perhaps not as exciting as recreating live dinosaurs from DNA extracted from ancient mosquitoes, but almost as amazing, is manipulating DNA in order to identify particular genes or alleles. Although creating entire organisms from a DNA sample is not possible at this time, recombinant DNA analysis is a powerful tool used in many different disciplines including human forensic science, diagnostic medicine, paternity testing, investigation of wildlife poaching, and plant and animal science. The later category, animal science, was the concern of this lab study.

DNA analysis has made it possible to isolate a gene or group of genes which represent a characteristic and to selectively breed for that characteristic. The American pig has been extensively bred to produce a more desirable pig i.e.., leaner and/or larger. One trait the American pig does lack is the ability to produce a high number of offspring known as prolificacy or fecundity but this trait is possessed by the Chinese pig. The Chinese breed produces approximately 36 offspring per year as opposed to the American pig's 22 offspring per year, however the Chinese pig is very fatty rendering poor quality meat. Therefore, it would be ideal to create an American/Chinese pig hybrid that possesses all of the American traits, but also expresses the high fecundity trait. Fecundity is controlled somewhat by genes or inheritance and the rest is environmental. There is a group of several genes with major effects on prolificacy, but its exact location is unknown at the present time. It is suspected that the gene IGF-1, interleukin growth factor, is correlated to fecundity.

To compare the DNA between American purebreds, Chinese purebreds, and the American/Chinese hybrids and link this IGF gene to fecundity, the DNA from the specimens had to be isolated and prepared for analysis. There are many different methods that can be used for the isolation and analysis of DNA. The method used in this lab study was restriction fragment length polymorphism (RFLP) analysis. An RFLP is an associated genetic marker detected because DNA segments alter in length, therefore the variation in lengths of Chinese pig DNA versus American pig DNA can be compared. Restriction enzymes cut the DNA fragments at specific sites from the long DNA strands.

MATERIALS AND METHODS:

Extraction of pig DNA

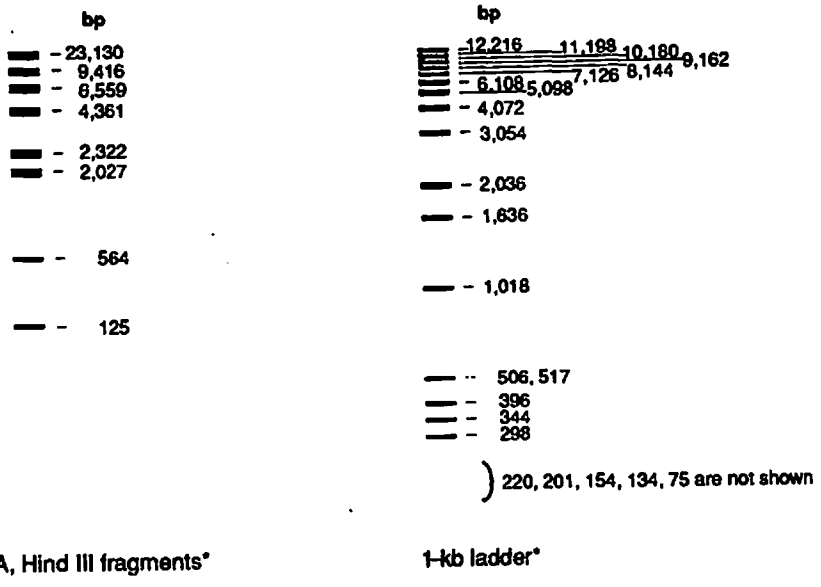
Approximately 1-2 cm of porcine tail from each of the specimens was digested with SDS and proteinase K. SDS is a detergent which initiates protein denaturation and proteinase K is a proteolytic enzyme that reduces the proteins to their component amino acids. The key to extracting nucleic acids, DNA, from complex molecules is the removal of the proteins. SDS and proteinase K aids this process. The extracting process was then continued using three different organic solvents. The first solvent to be applied was phenol, then phenol/chloroform, and last was CIAA. Deproteinization is more efficient when two different organic solvents are used rather than one. The last solvent, CIAA, is a phenol:chloroform:isoamyl alcohol solution which prevents RNase activity and helps to purify the nucleic acids. After extraction, the DNA was precipitated with ethanol by centrifugation. A centrifuge is a device that spins solutions at great speeds to separate materials of different densities at specific speeds. Sodium chloride was also used to keep

the SDS detergent soluble to prevent coprecipitation with the DNA. The DNA pellet was then resuspended in TE and stored at 4 C.

Electrophoresis of DNA

Electrophoresis is a process that takes advantage of the fact that DNA is negatively charged. The DNA fragments are separated with restriction enzymes and moved through an electric field. To prepare for this procedure, a liquified agarose solution containing ethidium bromide was poured into an electrophoresis gel box and allowed to solidify. The samples were loaded into the gel wells and moved from the negative to the positive pole. A tracking dye called "blue juice" was added to the samples before loading. Blue juice also acts as a weight to prevent the sample from floating out of the well. The gel was stained with ethidium bromide which intercalates between the base pairs. The gel is viewed on a UV light box. The DNA absorbs the UV irradiation and fluoresces orange-red.

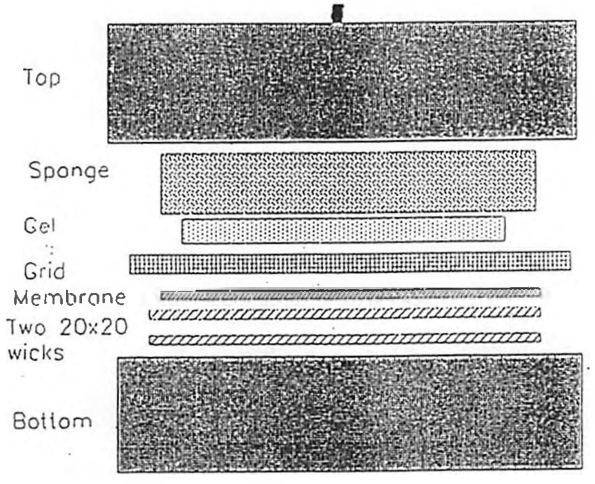
Factors affecting the distance traveled include: size of fragments, amount of voltage, and the concentration of the agarose in the gel. A smaller DNA fragment will travel faster than a larger one. Therefore the relative size of DNA fragments can be compared on a gel. The actual size of a fragment can be measured by running a size standard such as lambda DNA on the same gel. The sizes of the lambda standard fragments are already known. (See figure below). Six different gels were run using various combinations of purebreds and hybrids. The separated DNA fragments were then transferred to a blot by Southern blot technique.



Southern blotting

Electrophoresis gels are fragile and cannot be kept for a long period of time, therefore,

DNA is transferred to a nylon membrane. A nylon membrane blotted with DNA can be probed over and over again provided the probe from the previous hybridization is sufficiently removed or decayed. The southern blots were produced using a pressure blotter to transfer the DNA from a gel to a nylon membrane. The pressure blotter apparatus consisted of a vacuum, an airtight glass box, two pieces of wick paper, a nylon membrane, a grid, the gel, and a sponge. (See figure below). The vacuum creates a high pressure environment which allows for rapid transfer of DNA. The buffer is drawn from an upper chamber and elutes the nucleic acids from the gel and deposits them on the membrane as it passes through to the lower chamber.



The sponge was soaked in a sodium chloride and sodium citrate solution (SSC + H_2O), which is a buffered transfer solution. The gel was prepared by submerging it in an HCL solution, which increased the transfer efficiency. The HCL causes depurination which shears the DNA into smaller pieces. The gel was then soaked in denaturant which separates the double-stranded DNA into single-stranded DNA. Lastly, the gel was soaked in neutralizer. The gel was then placed in the pressure blotter and sealed tight. The pressure in the box was raised to approximately 75 psi by the pressure control center, or vacuum, and allowed to run for 40-45 minutes. By this time, the DNA should have been sufficiently transferred and was checked by placing the gel on the UV light box to look for residual DNA. The membrane was placed in an SSC solution to remove residual agarose before placing in a baggy and sealing. The membrane was then irradiated on the UV light box to fix the DNA. The Southern blot was then ready for probing.

Amplification of probe of porcine IGF-1

The probe for IGF-1 was sent to the lab already cloned into the plasmid vector pUC8. The clone was sent by Dr. Bertram Brenig from the Institut Fur Biochemie in Germany. The plasmid vector is double-stranded circular bacterial DNA. A restriction enzyme cuts the plasmid at a specific site and the IGF-1 coding region is inserted into the plasmid. This is done because bacterial DNA can be amplified greatly and therefore produce more of the IGF-1 coding region for experimentation.

The IGF plasmid, which is ampicillin resistant, was grown on ampicillin agar plates to obtain a pure isolated bacterial colony. An isolated colony was placed in LB broth to grow first and then placed in TB broth to grow a high yield. The LB broth and TB broth are Luria-Bertani medium and terrific broth respectively which contain bacto-yeast, bacto-tryptone, bacto-agar, and ampicillin and provide maximum growing conditions. The samples were then centrifuged to collect a bacterial pellet. The supernatant was poured off and the bacterial pellet was allowed to dry. The pellet was then resuspended in TE. A plasmid preparation procedure was performed. The IGF plasmid was then cut with the enzymes EcoRI and HinDIII. A small sample was run on an electrophoresis minigel to insure proper cutting of the DNA. At this point the minigel reflected that the cutting of the bacterial plasmid was not successful. The entire sample of IGF plasmid was washed with equal volumes of phenol and chloroform. Centrifugation was used to recover the pellet. The pellet was then resuspended in ethanol and potassium acetate, concentrated back into a pellet through centrifugation, dried, and once more resuspended in TE. A sample of the IGF was then redigested with EcoRI while another sample was digested with HinDIII. Only the digestion with EcoRI was successful so this enzyme was used to digest the rest of the IGF plasmid sample. The digested sample was then run on a midsize gel to prepare for electroelution.

Electroelution

The electroelution process is one method of isolating DNA from an agarose gel. The bands of probe DNA were physically cut out of the gel while viewing on the UV light box. These bands were then placed into the electroelution apparatus. Ammonium acetate was added to the apparatus to create a salt bridge. This aids in eluting the DNA from the agarose gel into a buffer.

Making the probe

A small sample of the probe DNA was added to TE and heated at 95 °C to denature the strands. Separating the strands can be achieved using a chemical denaturant or by using heat. Then added to the probe DNA was random priming buffer, nucleotides, radioactive dATP, and Klenow fragment. This was incubated at room temperature for two hours. Ammonium acetate, TE, ethanol, and tRNA were then added and the radioactive probe DNA was precipitated out. The pellet was resuspended in TE and the radioactive level was then measured. The probe was heated at 95 °C to denature the strands. The probe was then added to a hybridization solution and was ready to be added to the blot.

The random priming buffer assisted in the binding of the nucleotides. The nucleotides are cytosine, guanine, thiamine, and adenine. The nucleotide adenine, A, itself was not radioactive. The inner phosphate of dATP, deoxygenated adenine triphosphate, was labeled ^{32}P. The Klenow fragment is a DNA polymerase which constructs the nucleotides into a strand complimentary to the probe DNA. This process results in a radioactive probe complementary to the IGF gene sequence. The probe is then denatured to produce a single-stranded, radioactive sequence of DNA which will bind to the complimentary single-stranded IGF DNA sequence on the blots of the pig DNA.

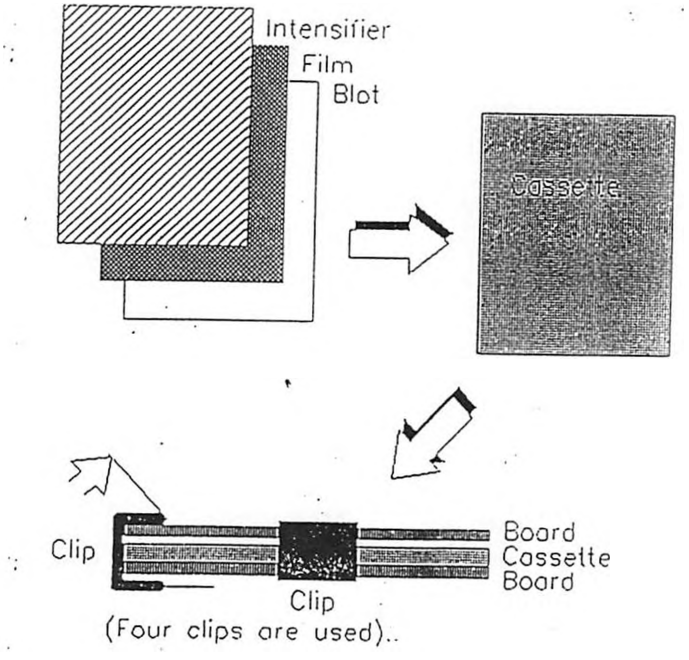
Probing the Southern blots

The blots were prepared for probing by soaking them and incubating in a prehybridization solution, which prevents non-specific binding. The hybridization solution containing the probe

was added to the blot and incubated overnight which also reduces non-specific binding of the probe to the membrane. After probing the blot, it was washed three times which facilitates the removal of the loosely bound probe. As the stringency of the wash solutions was increased, more nonspecifically bound probe was removed leaving only tightly bound complimentary strands. The washing also decreases the radioactive level of the blots. The blot was then placed in a new baggy and sealed tightly for autoradiography.

Autoradiography

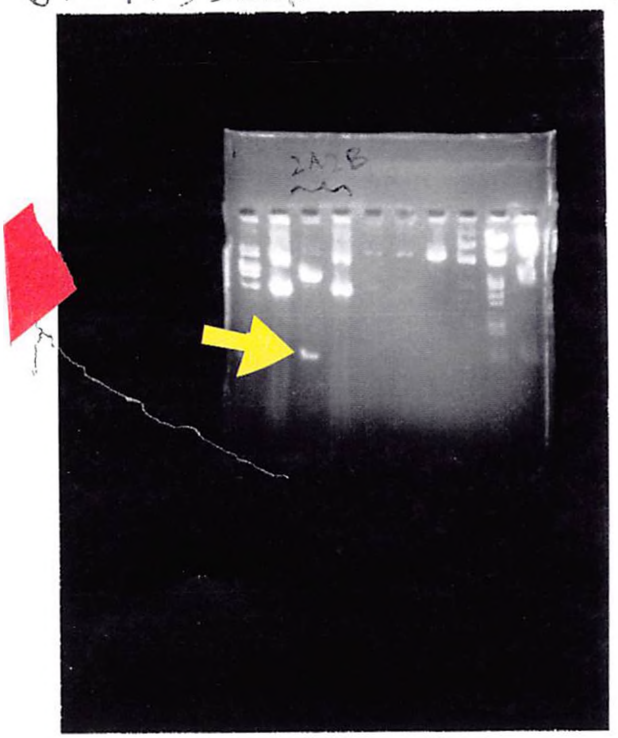
Detection of ^{32}P-labeled hybridization probes on blots is facilitated using autoradiography, in which a picture of the blot is taken. The blot was placed in an X-ray cassette in a dark room and then placed in a -80 °C freezer for four days. The film was then developed in an automated processor.



RESULTS

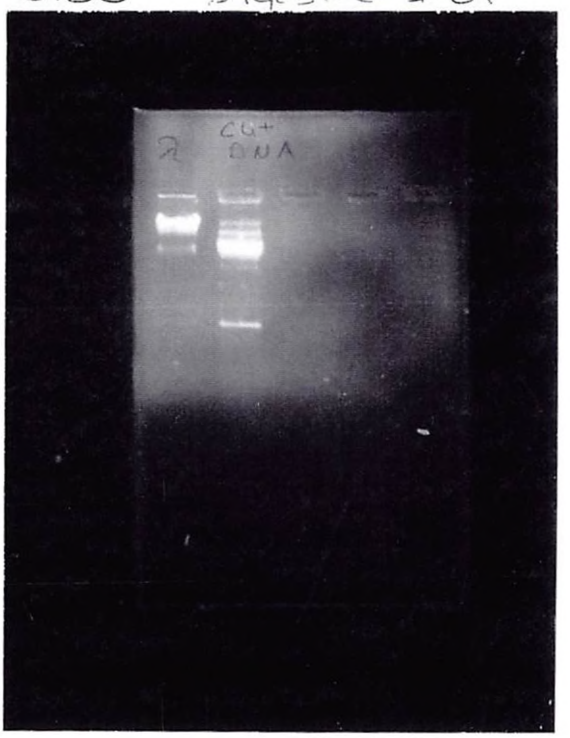
Electrophoresis Gels

6/29: 5 samples of cut and uncut DNA

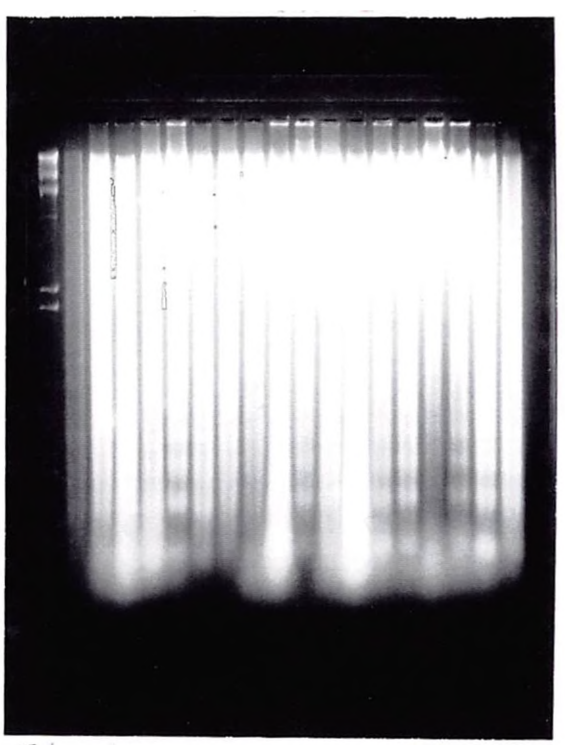


Arrow indicates band cut with EcoRI.

6/30 Digested IGF



DNA cut with EcoRI run along a size standard, λ.

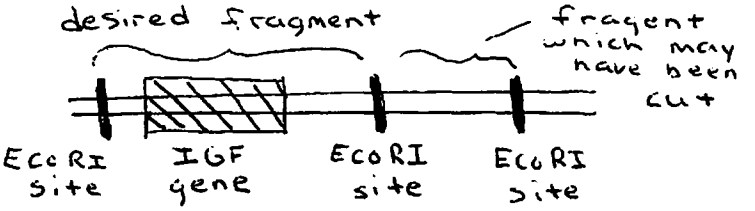


5/27/93; Blot V cut w EcoRV

← Blot containg DNA smears before being cut with enzymes and denatured.

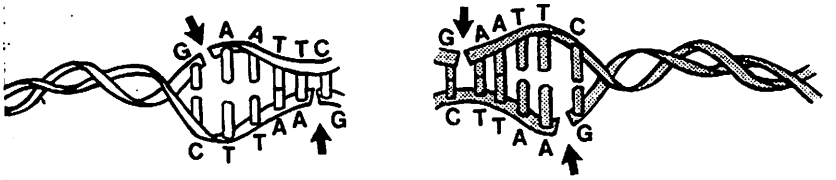
DISCUSSION

The probe was unsuccessful in binding to the IGF gene. One probable cause may have been that the restriction enzymes may have cut the DNA at a place other than the IGF gene site. Restriction enzymes cut DNA at specific sites on the genome. It is possible to have many sites specific to the same restriction enzyme on the genome. The restriction enzyme used could have cut the DNA at sites downstream from the IGF gene site. (See illustration).

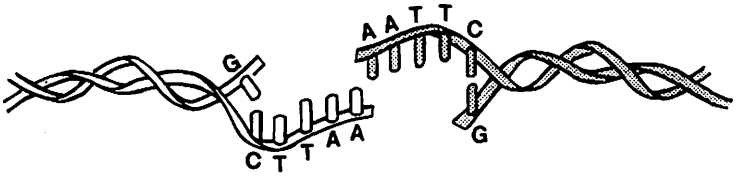


DNA strand containing IGF gene

Since the probe was probably cut at an irrelevant sequence, the sequence would not have been complimentary to the IGF gene sequences on the blot. Therefore annealing of complimentary sequences was not possible. The illustration below demonstrates the annealing process of complimentary strands creating a recombinant DNA molecule. If this probe had been successful, RFLP analysis would have been done. A similarity in the banding patterns between the Chinese pig and the hybrid pigs was the desired result. If this was the case, further study would include breeding the hybrid pigs which had the same IGF fragment length as the Chinese pigs and checking for the high fecundity trait. This correlation between fragment length and high fecundity would have to be tested over several generations to determine how tight the link is between the IGF gene and the high fecundity trait of the Chinese pig.



Restriction enzyme cuts both DNA molecules at the same sequence site



Fragments join at "sticky" ends

Recombinant DNA molecule