Separation and cloning of IGF-1R quality in cattle

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Abstract

Gene cloning, or molecular cloning, has several different meanings to a molecular biologist. A clone is an exact copy, or replica, of something. Molecular biologists exploit the replicative ability of cultured cells to clone genes. Recent advances in the Restriction enzymes technologies have opened up new opportunities in biotechnology and facilitate the way to isolate any gene from any source of cells. Many types of restriction enzymes cleave DNA away from their recognition site. By using the type III restriction enzyme, we provide evidence to show that an intact recognition site on the cleaved DNA sequesters the restriction. We isolated and cloned the IGF-1 R gene from DNA by subjecting DNA with one pair of restriction enzyme (*EcoRI + Hind*III) and followed by ligation and transformation with the suitable vector. The pair of restriction enzymes (*EcoRI + Hind*III) which subjected to DNA template before the ligation process was the only pair which had success to liberate IGF-1R fragment from the transformed vector rather than other two pairs of restriction enzymes (*EcoRI + Xho*I) or (*Hind*III + *Xho*I) although this two pair of restriction enzymes have recognition sites in the vector. Moreover we have studied the effect of DNA template size on the action of the restriction enzymes. Where less DNA size, more efficient of the restriction enzyme action. This study is rapid and useful new technology for isolation, cloning and sequencing the gene of interest to be ready for further investigations as gene transfection and/ or transfer.

Keywords: Restriction enzymes- DNA technology- IGF-1R- bovine

INTRODUCTION

Since the first convincing demonstration of somatic cell nuclear transfer by (Wilmut et al., 1997) and production of the first transgenic cattle, (Cibelli et al., 1998) there has been renewed interest in both understanding the concept of nuclear reprogramming and developing more efficient ways of producing animals by nuclear transfer. The production of transgenic animals by germ and somatic cell nuclear transfer have proven to be a more efficient method than other methods such as gene injection or sperm mediation. A wide variety of restriction –modification (R-M) systems have been discovered and characterized. They are classified based on their subunit composition, cofactor requirement and mode of DNA cleavage, (Pingoud and Jeltsch, 2001) . Restriction enzymes cleaving DNA in type I and type III classes are dependent upon ATP and *S*-adenosyl-L-methionine (AdoMet), (Murray, 2000; Rao et al., 2000). Type III enzymes require two inversely oriented, asymmetric, unmethylated recognition sites on the same DNA mole-cule to introduce one double strand break between the two sites. Type III restriction enzymes such as *NaeI* and *EcoRII* interact with two copies of their recognition seq-uence, one being the target for cleavage and the other serving as an allosteric effector. This contrasts with the orthodox type II restriction enzymes that cleave DNA within the recognition site. While essentially all restriction enzymes require the divalent metal ion, Mg^{2+}, for DNA cleavage, types I and III in addition require ATP and Ado-Met. The intervening sequence between these sites can be anywhere between 30 and 3000 bp in length, (Meisel et al., 1992). Physical interaction of enzyme molecules bound to both sites is essential for DNA cleavage to occur. The insulin-like growth factor (IGF) peptides (IGF-I and -II) mediate their effects through the type I IGF receptor (IGF-1R), while the IGF-binding proteins (IGFBP-1 to -6) modulate their interaction with the receptor. The IGF sys-tem is expressed in the uterus during the oestrous cycle and early pregnancy and is likely to play an important role in regulating the development of the embryo and uterus.

Insulin-like growth factors (IGF)-I and -II are single chain polypeptides which are structural analogues of proinsulin. IGF -I and -II promote DNA synthesis, cell proliferation and differentiation as well as having acute anabolic effects on protein and carbohydrate metabolism (Jones and Clemmons, 1995; Hossner et al., 1997).

We aimed to isolate and clone IGF-1R gene. A comparison between the restriction enzyme action on a small and large DNA segments was investigated.

MATERIALS AND METHODS

Isolation of different size of DNA from blood sample

Rapid Isolation of mammalian DNA (20 - 50 kb)

DNA (20-50 kb) was extracted from bovine blood sample according the method described by Sambrok and Russell, (2001) as follows: 300 µl aliquots of whole blood were transferred to two separate microfuge tubes. 900 µl of 20 mM Tris-HCl was then added to each tube and mixed. The tubes were incubated at room temperature for 10 min, followed by centrifugation at a maximum speed for 20 s.

The supernatent was discarded and the pellet (white blood cells) was resuspended in 600 µl of ice cold cell lysis buffer. Protinase K was added (3 µl) to each tube followed by incubation at 55ºC for 3 - 16 h.

Add 3 µl of 4 mg/ml DNase- free RNase to the cold solution and incubate for 1 h at 37ºC. Add 200 µl of potassium acetate solution and mix for 20 s then centrifuge for 3 min at maximum speed at 4ºC. Transfer the supernatant to a fresh tube containing 600 µl of isopropanol and mix well and centrifuge at 10,000 rpm for 1 min at room temperature. Transfer the supernatant and add to it 600 µl of 70% ethanol to percipetae DNA and mix well before centrifuge at 1000 rpm for 1 min at room temperature. Carefully remove the supernatant and allow DNA pellet to dry in air for 15 min. Dissolve the pellet of DNA in 100 µl of TE (pH 7.6) and stored at – 20ºC till analyzed.

Salting out method (100 kb DNA)

DNA was extracted from blood sample of bovine and this protocol is used to get (100 kb DNA size). 10 ml bovine blood was dissolved in EDTA and cell lysis buffer followed by centrifugation at 10000 rpm for 15 min at 4ºC. The pellet was dissolved using nuclei lysis buffer, 20% SDS and proteinase K. Saturated NaCl solution was added after the incubation and centrifuged to get the supernatant which was dissolved in ice cold absolute ethanol then centrifuged. The DNA was removed by using a heat- sealed Pasteur pipette and washed in 70% ethanol and let to dry on the air. DNA was dissolved in 200 µl TE buffer and stored at – 20ºC till analyzed.

DNA digestion and PCR program

Ten µl of DNA sample were incubated with 0.75 µl *EcoR1*, and 0.75 µl *HindIII* and 2 µl buffers of the two enzymes and 1.5 µl dd water at 37ºC for 3- h.

PCR amplifications were performed following the procedure of (Williams et al., 1990). The primer of IGF-1R gene (SEQUENCE SIZE: 556 bp DNA linear and accession number U33122).

Primer sequence (5' to 3') (Left: CCTGCGCAATGGAATAAAGT and Right: ATTGGGTTGGAAGACTGCTG) was designed by Primer 3 and Primer 5 program to get PCR product of 163 bp which was synthesized from (Takara compony- China) and was used in this work.

PCR reaction was carried out in a 25 µl in an eppendorf tube containing 25 ng of genomic DNA. Amplifications were performed in a Perkin Elmer 9700 Cetus thermal cycler which was programmed as follows: an initial denaturation step at 94°C for 2 min followed by 40 cycles with 94°C for 30 s, 55°C for 30 s 72°C for 30 s, final extension at 72°C for 10 min were carried out. The samples were cooled at 4°C. The amplified DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. The amplified pattern was visualized on a UV transilluminator.

Cloning of IGF-1R

The PCR product was purified and verified by agarose gel analysis for the presence of a single band for IGF-1R before proceeding with cloning and sequencing by using purification kit. (From Takara company- China) IFG-1R gene was ligated to PMD18T- simple vector (From Takara company- China) as follow:

In 25 µL in an eppendorf tube containing the following mixture (use ice box), (1 µl PMD18-T vector, 1.5 µl DNA fragment, 3.5 µl dd water, 5 µl solution I (ligation mixture) then incubate at 16°C overnight or at least 1 h.

The transformation for the ligated gene occurred by using LB Medium for overnight followed by plasmid extraction. To isolate our gene of interest from the plasmid after the ligation and transformation and to define the main pair of the restriction enzymes which can liberate our gene of interest we divided the extracted plasmid into three groups according to the type of restriction enzymes. We subjected the plasmid to three pairs of restriction enzymes as follow: 1- (EcoRI + HindIII, 2- EcoRI+ XhoI and 3- HindIII+ XhoI.

PCR program was carried out to all tubes after the digestion to define and isolate our gene of interest.

Sequencing of both IGF-1R fragment and sequence analysis

After the ligation and transformation in LB medium, we choose the plasmid which have IGF-1R gene and were sent to the sequencer (Sangon –China) to compare our results with that in gene bank.

RESULTS AND DISCUSSION

Molecular biologists exploit the replicative ability of cultured cells to clone genes. Gene cloning also enables scientists to manipulate and study genes in isolation from the organism they came from. This allows researchers to conduct many experiments that would be impossible without cloned genes.

Modern geneticists often use the same methods as Mendel and Morgan to identify genes by physical traits, or phenotypes, that mutations in them can cause in an organism. But today we can go even further. Using a broad range of molecular biology techniques, including gene cloning, researchers can now determine the precise DNA coding sequence that corresponds to a particular phenotype. This capability is tremendously powerful, because discovering the gene responsible for a trait can help humankind understand the cellular and biochemical processes underlying the trait. In the present study we aimed to isolate and clone IGF-1R gene from bovine blood to be useful for further applications to do more focusing about this useful gene.
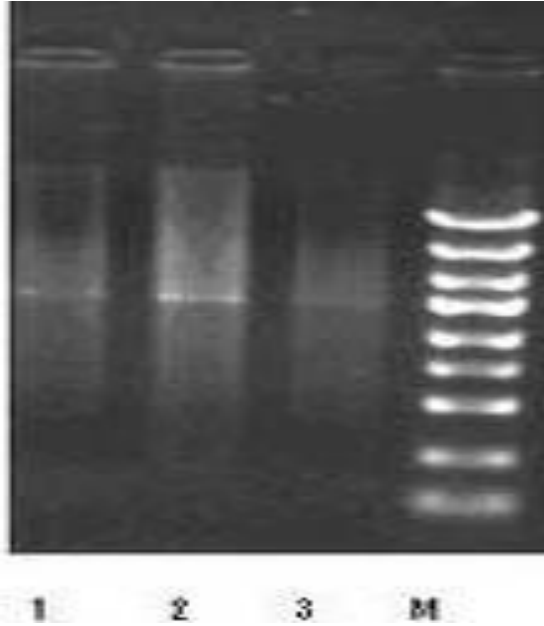
Figure 1. Showing the DNA after random digestion with the restriction enzymes (*EcoRI* and *HindIII*) lanes 1 and 2 are small DNA (20- 50 kb) and lane 3 is large DNA (100 kb) and M is 5000 bp marker.

The insulin- like growth factor (IGF) system, consisting of two growth factors (IGF-I and IGF-II), two IGF receptors (IGF-1R and IGF-2R/cation- independent manose 6-phosphate receptor, M6P-R) and six IGF binding proteins (IGFBP1-6), has been characterized as an important regulatory system for controlling tissue growth and development in vertebrate species (Jones and Clemmons, 1995).

The importance of growth factors and growth factor receptors has been studied indirectly by demonstrating that receptor activation can stimulate cell proliferation and embryo differentiation. It was demonstrated that insulin, insulin-like growth factor (IGF)-1, and IGF -2 induce expression of HIF-1 ⍺, which is required for expression of genes encoding IGF-2, IGF-binding protein (IGFBP)-2 and IGFBP- 3. The IGF peptides (IGF-I and -II) mediate their effects through the type 1 IGF receptor (IGF-1R).

In mammals, IGF-I is thought to play a pivotal role in mediating the effects of growth hormone (GH) on tissue growth during postnatal development (Dupont and Holzenberger, 2003).

In liver, expression of the IGF-I gene was low throughout embryonic development before increasing dramatically (8 fold) by 3 weeks pH. This is consistent with several observations concerning the regulation of the hepatic IGF-I gene. First, it has been previously noted in mammalian species that liver -derived IGF-I in circulation plays an important role during postnatal development in mediating the growth-promoting effects of GH (Dupont and Holzenberger, 2003).

In study of Robins (2000), the expression of the IGF sy-

stem in the bovine uterus was determined throughout the oestrous cycle and on day 16 of pregnancy, (Robinson et al., 2000). IGF-I and -II have been shown to stimulate embryonic production of IFN- *in vitro* and it is likely that IGF- I and -II play an important role in development of both the embryo and uterus during early pregnancy, (Wathes et al., 1998). The actions of IGF-I and -II are mediated through the IGF type 1 receptor (IGF- 1R). IGF-1R has been localised to the pre-implantation embryo, (Watson et al., 1992) and to the glandular epithelium of the ovine uterus (Stevenson et al., 1994; Reynolds et al., 1997a) supporting a role for IGFs in embryonic and uterine development. More recently, the presence of the methyl donor AdoMet has been shown to be mandatory for restriction, (Bist et al., 2001). The role of exonucleases in processes such as DNA replication, recombination and repair has been well documented in various organisms, (Shevelev and Hübscher, 2002).

We studied effect of DNA size and their action on the process of the cloning. We found that the small size of DNA (20- 50 kb) is easily digested, isolated and cloned to give us pure IGF -1 R fragment than the large size (100 kb DNA) (Figure 1, 2 and 3).

Moreover, we studied effect of the type restriction enzymes and its role in the cloning. The pair of the restriction enzymes (*EcoRI* and *HindIII*) which used for random digestion of DNA template before the ligation and transformation processes was the only pair of the restriction enzymes which liberated the fragment of IGF- 1R after the ligation and transformation with the suitable vector. We did not get IGF-1R fragment with the other two pairs of restriction enzymes (*EcoRI* + *XhoI* or *HindIII* + *XhoI*) after the ligation and transformation processes.

Using molecular cloning techniques, we have identified and sequenced a portion of the bovine IGF-I gene corresponding to the entire coding region and the sequencing result demonstrated high homology (>95%) with that in the gene bank (556 bp DNA linear and accession number U33122).

Restriction enzymes have the ability to cut DNA molecules at very precise sequences of 4 - 8 base pairs called recognition sites. They naturally occur in many bacteria as a defence mechanism against bacteriophages (viruses that infect bacteria). The restriction enzymes in the bacteria were able to cut the viral DNA, usually at least at several locations, thus rendering the viral DNA inactive so it couldn't infect the bacterium.

Purified forms of these restriction enzymes are now used as tools to cut DNA. Scientists use over 400 restriction enzymes that recognize roughly 100 recognition sites as a means to isolate sequence and manipulate individual genes taken from any cell (including humans). The sites where the fragments of DNA are cut may result in overhanging "sticky ends". Pieces with similar sticky ends can be joined together using the enzyme *DNA* ligase. Hybrid DNA from different sources (or species) may be produced this way and such DNS is known as recombi-
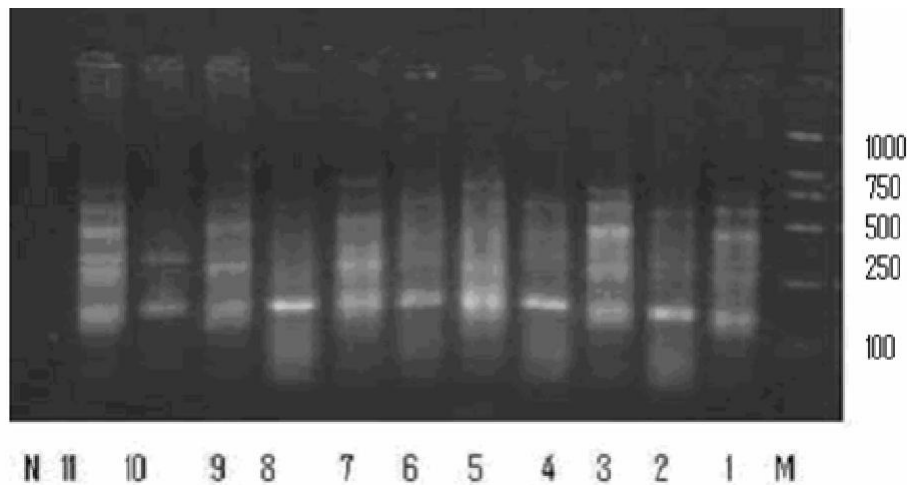
Figure 2. Showing the analysis of agarose gel electrophresis of PCR products for IGF-1R which were isolated from different size of DNA where M is 2000 bp marker, lanes, 2, 4, 6, 8 and 10 used from small DNA size (20 - 50 kb) and lanes 1, 3, 5, 7, 9 and 11 used from large DNA size (100 kb) and N is negative control.
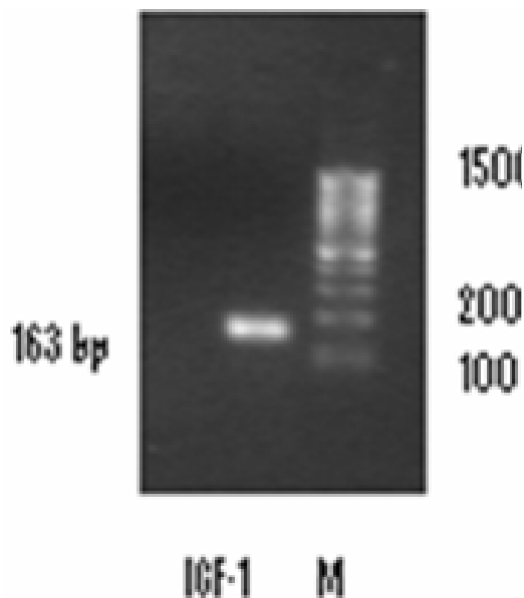

Figure 3. Showing the analysis of agarose gel electrophresis for IGF-1R (163 bp) after the purification.

nant DNA.

Conclusion

Our study was a new way to isolate and clone an important gene related to the fertility of bovine as IGF-1R from DNA using easy and rapid method Moreover we studied the effects of DNA size on the action of the restriction enzymes.

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