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Course Manual

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TRANSGENIC FISH

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Definition

Transgenic animal is defined as an animal that has been become transformed following the introduction of a novel gene in to its DNA. Gordon et al (1980) were the first to produce transgenic animals leased on the microinjection of cloned DNA into pronucleus of the fertilized eggs at the arc cell stage. Palmitev et al (1982) INTRODUCED GH gene into mice and achieved dramatic increase in growth of the animals and the successful transgenic animal has been produced. Thus a 'giant' mouse was produced – normal 29gms- the giant mouse grew up to 44gms. Sadly the giant mouse died prematurely.

However, Introduction of novel genes and production of transgenic fishes were very slow- it was only in (1985) Zhu et al introduced novel genes into gold fish (CARASSIUS AURATUS L 1758). The gene introduced was growth hormone gene. Gill et al (1985) showed that chicken growth hormone, and bovine growth hormone accelerated growth in Pacific salmon. Soon fish growth hormone gene was isolated and expressed. Now many laboratories are working in this area.

2. Advantages of fish as transgenic animals:

i). Large number eggs are available.
ii). Can be fertilized under controlled conditions.
iii) Need not to return to the female reproductive tract for completion of development as in the case of mammals.
iv) Availability of a large number cloned (c DNA) genes.
v) Transgenic fishes are recognized as the superior system for examining the molecular development of early vertebrate development.
vi) Transgenic fish may play an important role in aquaculture.
vii) There are no ethical considerations, which restrain gene manipulation on higher animals.
viii) Transparency of embryos in many species.
ix) Haploid, Polyploid individuals are available.
x) Gynogenetic and androgenetic individuals are available to study the system.

3. REQUIREMENT FOR PRODUCTION OF TRANSGENIC FISHES:

Three steps are involved in the production of transgenic fishes

a) Specific recombinant DNA construct must be prepared having the gene of interest or the regulatory element in it.

b) The recombinant DNA must be delivered to the nucleus of the cell of the developing embryo/ fertilized egg in order to be distributed to all the tissues of the fish.
c) Since all deliveries of the trangency will not be effective and not all constructs will behave in the manner that was desired, a screening process must be established.

3.1 .**VECTORS**

For producing transgenic fishes the genes should be cloned in to the expression vectors. Expression vectors have regulatory sequences that determine where, when and the level at which the transgene will be expressed. All the vectors so far used in transgenic fish studies show the following general factors. They are all plasmid vectors can be replicated to the level of 500 to 2000 copies per cell with selectable and unique restriction sites.

6.6Kb Bam H1 and EcoR1 fragment used for microinjection.

This plasmid contains the mouse metallothionine gene and rat growth hormone gene having the necessary regulatory elements and gave good expression. The use of heterologus GH genes was found to be a problem, such as improper splicing and translation. Further the use of heterologous genes was mainly due to the lack of availability of suitable piscine sequences. Many workers have developed all fish vector – Liu and coworkers used Carp beta actin gene enhancer –promoter with Ploy (A) addition signal from salmon.
These vectors expressed in all tissues. Another group has (Hew et al)
constructed all fish expression vectors using antifreeze protein gene promoters-This vector did not express in all the tissues. Since beta actin promoters are stronger than AFP promoters constructs with trout metallothionine gene are also available but they are not preferred because heavy metals such as Zinc are to be used for inducing the transcription of the genes.

The vector should contain introns, enhancers, boundary regions, control regions in addition to promoters for the proper expression (Palmitiv et al 1991).

3.2 REPORTER GENES:

There must be suitable reporter genes to see the presence of the transgene in the various tissues of the animals. Reporter gene is defined as a gene whose products detects or marks the cells, tissues, organisms that express the gene from those that do not. Reporter genes isolated from prokaryotes, E.coli, are used in fishes – Lac Zgene , Cat(Chloramphenicol Acetyl transferase gene). Recently luciferase and green fluorescent protein are used.

3.2.1. **LUCIFERASE:**

This is the most sensitive and one of the simplest reporter systems adopted in transgenic fish. This gene is isolated from firefly PHOTINUS PYRALIS. Previously they used to cell/tissue homogenates where the reaction was started by the addition of luciferin substrate containing Acetyl CoA and ATP. The enzyme activity was monitored by photon emission by scintillation counter.

In 1990 TAMIYA et al demonstrated for the first time that the incubation of living MEDAKA embryos in a solution of luciferin resulted in the penetration of the substrate in to the living tissues thus allowing the detection of the expressing embryos by using a photon counter or by if the embryos are placed on a very sensitive X-ray film they can be detected.

Advantages:

It has many advantages over other reporter systems.

a) Luciferase is a simple polypeptide, which does not require post translational modification.
b) There is no endogenous background of any sort.
c) Shows linear activity upto 10^6 units.
d) The half-life is only 3 hours.
e) It is also a quantitative assay if tissue homogenate are used.

3.2.2 **GREEN FLOURESCENT PROTEIN:**

This a novel gene isolated from a jellyfish AEQUOREA VICTORIA. Light is produced when calcium binds to this protein. GFP has 238 amino acids absorbs blue light at 395 nm and emits green light at 509 nm. This fluorescence is stable. Use of this as a reporter gene has many advantages.

a) It does not require external substrate over it is to be irradiated by blue light. So not limited by availability of the substrate.
b) So gene expression can be monitored in living cells / animals.
c) The substance is not toxic to living cells/ animals.
d) It is more useful in embryos, which are transparent as ZEBRA fish embryos.
e) Since this protein is persistent even in formaldehyde fixed tissues it can be examined later also.

3.3 **GENES OF INTEREST:**

GROWTH HORMONE GENE

It is a circulatory hormone, which induces growth. It also facilitates seawater adaptation and stimulates gonadal steroidogenesis in salmon. Experiments have shown that transgenic pacific salmon was 11 times heavier than control (highest was 37 times heavier than control). However, There was an increased mortality in transgenics.

ANTIFREEZE PROTEINS

This protein was found in fish inhabiting cold water. They lower the freezing point of the plasma. They are largely synthesized in liver and released into the circulation.They are classified into three types depending on the amino acid sequences. Attempts are being made to increase tolerance of salmon, which lack AFP, and this can allow them to be cultured in north Atlantic waters. However AFP can give general cold tolerance.

DISEASE RESISTANCE

Fish has poorly developed antibody dependent immunity. The antibacterial enzyme lysozyme found in blood, mucous, kidney and lymphoid tissues play a role in nonspecific line of defence. Rainbow trout lysozyme if potent inhibitor of gram negative bacteria such as Vibrio anguillarum, V.harveyi and Aeromonas etc.

METALLOTHIONINE (MT):

It is a regulatory protein. It can be induced by $ZnCl_2$. This can be used for monitoring pollution of water. They're also other genes that can be used for developing transgenic fish.

3.4. **METHODS OF GENE TRANSFER:**

For production of transgenic animals the gene should be transferred successfully. There are many ways by which the genes can be delivered to the cells. The most important are Microinjection, Electroporation, Sperm delivery, Lipofection, Particle bombardment (Gene guns)

3.4.1: **MICROINJECTION**

This technique was originally was developed for mouse eggs and is used in fishes also. This is very efficient in mouse because of smaller number of eggs and small size of egg and the pronucleus are visible and the eggs are not surrounded by hard shells. Fish eggs are large 1-7 mm in diameter, (300 to 30,000 times larger than mammalian eggs). Cellular / nuclear volume ratio in mammalian egg is 20 whereas in fish egg is >100,000.Hence seeing the pronucleus is very difficult. Further the rapid onset of cleavage and hardening of the chorion. In spite of the difficulties this procedure is straight forward and attempts are made to adopt this procedure to suit fish eggs. Some species has softer chorion such as Catfish, Zebra fish. Small volume of the solution 1-2 nl of DNA containing $>10^7$ copies should be injected. The survival rate after injection is 80-80%. High level of mosaicism (90-99%) of the integration is noticed suggesting that the integration has not occurred efficiently. Further the first cell division is after 30 minutes after fertilization. So it is difficult to handle large number of eggs. Hence there is an advice for developing efficient procedures for mass transfer.

3.4.2: **ELECTROPORATION**

Electroporation is a technique based on exposing the cell membranes to high intensity of electric field pulses. As a result specific regions of the cell membranes are temporarily destabilized. During the stabilization the cell membrane is highly permeable to exogenous molecules present in the surrounding media. This method has been successful for gene transfer to prokaryotes and eukaryotic cells in culture. Since 1990 it is gaining favour among research workers. INOVE and his coworkers demonstrated that electroporation of medaka+ eggs resulted in 25% survival and 4% of those had transgenic fishes (800ml of solution having 80mg DNA)They used 100ng DNA /embryo capacitor driven 50milli seconds pulses at 750V/cm. In Zebra fish they used 0.1milli second pulses of 125V/cm for batches of 200 eggs. However each experiment has to be standardized as per the requirement to obtain optimum success.

3.4.3: PARTICLE BOMBARDMENT (GENE GUNS)

High velocity micro projectiles have successfully used to permeate the thick sell walls of certain fish eggs to deliver the transgenes. Primarily it was used in plants to transform chloroplasts. A thin coat of DNA is coated on to the surface of the 1µm diameter tungsten or gold particles by precipitation with calcium chloride. The beads are placed on the end of the plastic bullet (macro projectile) in the barrel of the gun specially designed for this purpose. The eggs are placed in suspension at the end of the barrel. The particles are sent towards the eggs by high speed and hits the cells. The barrel of the gun and the specimen chamber have to be evacuated, otherwise the speed of the particles are reduced. This technique was used for Zebra fish, Trout 75% survival and 5% transgenics were found. This is also a promising technique.

3.4.4: **SPERM CARRIERS**

Lavitrano showed that the mouse sperm could be used to convey externally cloned transgenic DNA into recipient eggs. Many fish workers have attempted to use this technique. This technique works in fish also. However, there was no evidence of chromosomal incorporation or successful F1 progeny. A modified procedure was developed ie sperm were electroporated, so that transgenes were internally confined to the sperm and carried on to the eggs with out injury. This also works in some case but not successful

3.4.5: **LIPOFECTION**

Here synthetic lipid vesicles encapsulate nucleic acid or nucleic acid protein complex and permit their uptake in to cells following fusion of the vesicles with the plasma membrane. Dechorionated catfish eggs were subjected to lipofection. Depending on the liposome/embryo ratio transformation frequencies were obtained resulting in the transgenic animals. Success ranged from 60 to 80%.

All the methods show clearly that there are two sides for expression. One is delivery and the second is expression. For successful production integration of the transgene must take place such that it is stable.

4. **DETECTION OF TRANSGENE**

After the introduction of the transgene the next question is to find out whether the gene has gone on to the system. There are various ways and means to detect this. Most of them used southern blot and northern blot to detect the transgenes using a suitable probe. (Restriction digest, gel electrophoresis, Filter transfer, labeled nucleic acids and probing) Then northern blot (dot blot) shows whether the DNA is synthesizing the mRNA and expressed. Western blot – see whether the desired protein is synthesized. PCR-through PCR also it is possible to detect small tissue like fin or other–template DNA then primer PCR.

5: **FATE OF THE INJECTED DNA:**

The transgene may be present in the animal but it may exist in various forms. It may not be present in the form, which is required for permanent transmission from one generation to another.

5.1: **DELIVERY OF DNA**

Transgenic DNA is generally delivered

a) super coiled plasmids containing both vector and transgenic DNA.
b) Linear DNA without vector DNA.
c) Concatemeric DNA of the transgenic DNA.

In general transgenic animals will not have bacterial vector. Therefore only transgenic DNA is injected. The linear DNA was better incorporated compared to circular DNA. Further the regulatory elements of prokaryotes are not essential for the fish. Further transgenic requirement emphasis that transgene material should not be evolutionarily distant (They should be close)

5.2: **MOST OF THE DNA IS DEGRADED**

Since cytoplasm is rich in exo and endonucleases most of the DNA degraded. However the digestion is not immediate.

5.3: **SOME COPIES OF THE DNA MAY PERSIST FOR LONG TIME BUT WITHOUT INTEGRATION:**

It has been shown that the DNA is replicated and undergoes conformational changes and only after many days the DNA disappeared. The DNA persist for a long time and get distributed to many tissues, such genes form concatamers.

5.4: **INTEGRATION AFTER PARTIAL DEGRADATION:**

Since degradative enzymes act on the DNA it is possible that a portion of the sequence may be lost before integration.

5.5: **INTEGRATION OF ONE COMPLETE COPY OR MANY COPIES:**

There is also a possibility that the whole of the DNA is integrated. However the precise location of the integration may not be known. Sometimes they may persist as pseudochromosome where centromere is in the transgene.The integration may be at many sites or at one site as concatamer.

5.6: **INTEGRATION AFTER SOME ROUNDS OF CELLDIVISION:**

This will lead to production of mosaics either because they are integrated in different sites at different tissues or integrated in some tissues and not in others. The mosaicism will lead to lack of integration into germ line cells and hence cannot be transferred to germ cells.

5.7: **INTEGRATIONWITHOUT EXPRESSION:**

Normally the integrated genes are expressed. Due to inactivation through DNA mehtylation or lack of satisfactory promoters or integration closed to heterochromatin the gene s may not expressed even though integrated.

5.8: **APPROPRIATE EXPRESSIONS:**

The desired result is the appropriate expression. In some cases it must tissue specific but in some cases it need not be. This will result in the phenotypic expression. This fact can be assayed by protein, or immunology or RNA blots.

5.9: **INAPPROPRIATE EXPRESSION:**

Expression will be appropriate if they are expressed in correct tissue and time.

5.10: **GERMLINE TRANSMISSION:**

The final goal of most transgenic work is the germ line transmission many worker were able to achieve this.

5.11: **CONCATAMERISATION:**

This is found just after infection of DNA of both in linear and circular. This may be [illegible] large amounts of DNA infection may result in this.

5.12: **REPLICATION:**

In fishes it is often found that the infected DNA, irrespective of the DNA sequence replicate in an extrachrosomal way. This correlates with the rapid DNA synthesis that takes place in the developing embryo.

5.13: POSITIVE EFFECTS:

The position of integration of the transgene in the chromosome shows influence /effect on the expression of the gene –called position effect. This accounts for the copy number and the level of expression. This is very obvious in transgene, which not does not

contain strong promoters/silencers. The inclusion of introns in the transgene has been found to enhance the expression and laso copy number dependent expression. Certain DNA sequences such as present in the boundaries of chicken lysozyme gene, hsp 70 genes when attached to the transgene the expression are more uniform.

5.13: **DNA METHYLATION:**

Transcription of RNA ploymerase II genes can be inhibited by DNA methylation. Novel genes upon integration become methylated and consequently inactivated. The mechanism is not clear. However, integration disrupts the normal chromosomal structure. If the embryos are treated with methylation inhibitor (5-azacytidine) better expression is found.

6: **FUTURE OF TRANSGENIC FISH:**

What is the outcome of the exercise?

6.1: **GROWTH ENHANCEMENT:**

This was one of the advantages **of** producing transgenic fish. Pacific salmon showed a dramatic growth increase. There are other fishes, which show enhanced growth such as carp, trout, and catfish. The aspects of growth

a) Initial fast growth so that they attain sexual maturity earlier.
b) Enhanced growth as adults –good for marketing.
c) Improved feed efficiency.

When we use Promoter such as viral or meatllothionine they are not accepted by the public.

6.2: **COLD TOLERENCE:**

In western countries and In our countries places like Himalayas and hill region people are interested in growing fish in cold areas. So fish should be able to withstand cold. So AFP genes are injected they may be able to withstand cold and grow better.

6.3: **PRODUCTION OF IMPORTANT PHARMACEUTICAL PROTEIN IN FISH:**

Use of fish as a bioreactor.

6.4: **IMPROVED DISEASE RESISTANCE:**

Fish are prone to viral and other diseases. Antisense RNA or Lysozyme genes it would be possible to produce fish, which show some resistance to diseases.

6.5: **STUDY OF VERTEBRATE DEVELOPMENT:**

Fish owing to its easy handling, short generation etc can be used as a model organism to study vertebrate development. To study enhancer/promoter / silence activity genes from other organism, mutageneis etc.

FURTHER APPLICATION OF TRANSGENIC FISH:

a) Better understanding of development, growth, gene regulation in fish and the reproduction process.

b) Improved economics of fish culture.
    - Improved feed conversion efficiency.
    - Improved cold resistance.
    - Improved freeze resistance.
    - Improved disease resistance.
    - Improved fecundity of brood stock.
    - Utilize low cost feed
c) Fish as bioreactors.
    - Production of medically important compounds.
    - Production of commercially important non-medical compounds.
d) Production of tailored fish.
    - With improved flavour.
    - With improved color, texture etc
    - With fatty acid composition.

Fish, as a transgenic animal will contribute more to the service and economics than any other organism because of its suitability and easy to handle.