# AN OVERVIEW OF GENE CLONING

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Gene cloning has been given varying names from genetic engineering to recombinant DNA technology and it has been attacked by various groups on religious, moral or ethinic grounds. It is defined as the isolation of individual genes by generating recombinant DNA molecules, which are then propagetd in a host cell which produced a clone that contains a single fragment of the target DNA.

The foundation for this field was built by numerous people, from Gregor Mendel's initial work on inheritance, to elucidation of the structure of DNA by Watson and Crick. The real spark plugs came in 1967, when DNA ligase was isolated followed by isolation of the fisrt restriction endonuclease in 1970. These enzymes act like a molecular "glue" and "scissors" respectively allowing DNA to be cut and joined. This lead to the creation of the first recombinant DNA molecule in 1972. Since then the possibilities have opened up with the main areas of application being

(i) basic research on gene structure and function
(ii) production of useful proteins
(iii) generation of transgenic plants and animals

The next big question is how do we clone genes and what is all the fuss about?

**Techniques of gene cloning**

There are four basic steps involved in gene cloning.

I- Choosing the source of DNA for cloning
II- Production of DNA fragments, which can be joined to vectors, from genomic DNA or mRNA
III - Insertion of fragments into vectors and vectors into the host organism for replication and thus amplification of the sequence.
IV- Analysis of the products for selection of the required sequence.

**Step I**

The decision lies between the choice of genotic DNA, which may contain extra DNA sequence responsible for control of expression or just repetitive sequences, and messenger RNA (mRNA). Ultimately the source is decided by the requirements of the study (for isolation of a single gene, mRNA would be the best choice e.g for isolation of an enzyme in the urea cycle, liver cells are chosen and mRNA extracted (as seen in the diagram)

**Step II**

mRNA cannot be cloned directly so it must be converted to DNA, known as complementary DNA (cDNA), by reverse transcriptase. Virtually every mRNA molecule has at its 3' end a run of adenine nucleotide called a poly A tail. Short oligonucleotides containing 12-20 deoxypyrimidines are mixed with the tails where they act as primers to reverse transcriptase. Alternatively, short oligonucleotide fragments 6-10 nucleotides long, made up of possible sequences are used as primer in a method termed randomly primed cDNA synthesis.

Reverse transcriptase creates a double stranded cDNA-RNA hybrid from which the RNA is removed by alkaline digestion. The single stranded cDNA is then rendered double stranded using DNA polymerase, and the product treated with another endonuclease, S1 nuclease to destroy the hairpin loop.

**Step III**

The double stranded cDNA molecules are then inserted into a plasmid or phage vector by (i) blunt end ligation (ii) use of linker molecules or (iii) homopolymer tailing.

Blunt end ligation involves the joining of cDNA fragments with DNA ligase. The blunt ends arise from filling in of the protruding ends of DNA fragments using DNA polymerase, or they result from the action of S1 ligase. It requires apropriate concentration of cDNA and vector DNA for optimal linkage to occur.

Linkers are self-complimentary oligomers that contain a recognition sequence for a particular restriction endonuclease creating sticky ends. These ends hybrize with the corresponding sticky ends created when the plasmid vector is opened using the same restriction endonuclase. The procedure results in the formation of an intact plasmid and in the formation of a recombinant plasmid containing inserts of cDNA. Included among the recombitant plasmids should be some which contain DNA synthesized from the relevant mRNA.

Homopolymer tailing, the most popular method, involves the distruption of the vector with a restriction endonuclease creating sticky ends. The 3' ends are lengthened by the addition of homopolymers of dA, dT, dG or dC. The cDNA molecule is also lengthened using the complementary nucleotide to the vector. The two fragments are annealed producing a recombinant molecule with the restriction site on each end.

The method of insertion of recombinant vectors into the bacterial host is dependent on the vector type. Plasmids are taken up by bacteria' hosts in a process known as transformation. *Escherichia coli (E.coli)* is the usual host and the plasmids must first be soaked in ice cold calcium chloride to make them competent. They are mixed with *E. coli* cells and incubated in ice for 20-30 minutes and then heated for 2min at 42°C. This is followed by incubation in a nutrient broth at 37°C for 60-90min to allow stabilization to occur. The process however, is inefficient and requires large amounts of vectors. Bacteriophage vectors are introduced to host bacterial cells in a process termed transfection. Following transformation or transfection the *E.coli* is then grown on agar plates. Introduction of recombinat vectors into non-bacterials cell has led to the development of techniques such as electroporation, microinjection and biolistic delivery.

**Step IV**

At this stage we end up with hundreds of thousands to a million bacterial colonies or phage plaques each containing a cloned

DNA fragment fragment, distributed on a set of agar plates. This constitutes a library. To identify the required sequence a combination of section and screening is required. Selection may involve the use of antibiotic resistant vectors planted on agar containing those antibiotics. This prevents the growth of bacteria which does not contain the recombinant plasmid. Selction can be achieved with the use of any of

(I) Hybridization with a nucleic acid probe

(II) Immunologic Screening

In both methods, a copy or replica of the library is prepared on nitrocelluose filters or nylon membranes. This process transfers a proprtion of each colony or plaque to the nitrocellulose paper, and is done in such a way that the pattern of plagues on the original plate is maintained on the filters.

In the former methods, a nuclein acid probe may be produced from prior knowledge of the amino acids sequence sought or more often from knowledge of the amino acid sequence of the protein expressed. In some cases part of the gene may already have been cloned and this can be used to search for clones that contain additional sequence flanking the starting clone. Hybridization between probe and cloned gene will occur if screening is carried out at low temperature and low salt concentrations. The probe may either be cDNA, genomic DNA or oligonucleotides which are radiolabelled, and light up (by autoradiography) colonies containing the cDNA sequences. The chosen clones can be isolated, grown and the DNA sequence determined.

**A discussion of gene cloning would not be** complete without metinong, at least in passing, the polymerase chain reaction. A technique devised by Kary Mullis and allows the selective amplification of DNA sequences. Simply put it involves repeated seperation of DNA strands by heat in a mixture of nucleotides and DNA polymerase. It results in an exponential increase in double stranded DNA fragments at the end of each cycle.

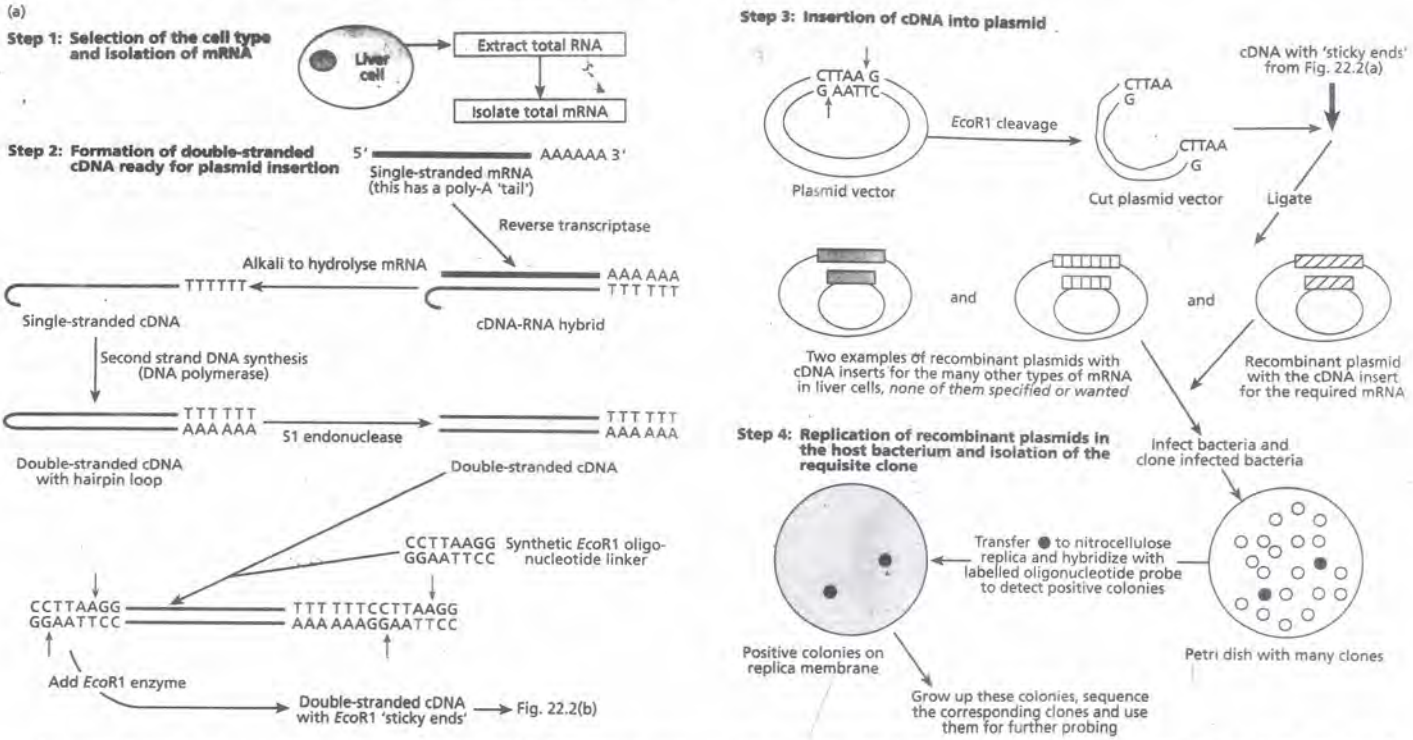
In the wake of the announcement of the completion of sequencing of the human genome, limitless possiblities abound. When the Roslin institute presented Dolly to an unsuspecting world, the public and biologists were shocked and many still debate how, and even whether the cloning technology that created Dolly should be applied to humans.

Hairs on neck still bristle when human cloning is mentioned. It stirs up feelings that scientists are going far in "meddling with nature" or that they are cheapening human life. Many religious groups also harbor specific objections because cloning is dependent on human embryos.

Yet there now seems less to worry about than first appeared. Only an extreme fringe would want to use cloning to make copies of people and such uses should be banned where they 're not already. But may researchers see enormous potentials in "therapeutic cloning" - the notion for growing tissues for patients that are genetically identical to their own. Example, neural cells could be made for people with Parkinson's disease; new muscles for those with ailing hearts; and later perhaps even whole organs might be grown, all free from the threats of tissues rejection.

To make Dolly, the Rosling researchers used the nucleus from an egg from a six year old Ewe. Astonishingly, the proteins in the egg's cytoplasm stripped the udder cell of its genetic control and returned its DNA to an embryonic state. The resulting cell then began to divide like a normal embryo

On the other hand, for therapeutic cloning researchers want to fuse a denucleated egg with a patient's cell and let it grow for a few days. they would then extract the stem cell and use them to grow the required kinds of tissues.




An example of a cloning strategy for preparing cDNA for a liver specific protein. In the final stage, the Escherichia coll restriction endonuclease I (EcoR1) enzyme cleaves the double stranded cDNA at the points indicated by the arrows thereby generating sticky ends" The double standed cDNA is then ready for insertion into a plasmis. (b) The remaining stage in the example of a cloning strategy for preparing cDNA for a liverspecific protein. cl;ones with required cDNA insert O other clones.

This seems an important use for human cells. Thousands of surplus embryos produced for IVF are detroyed every year. So objection to therapeutic cloning on the ground that embryos should not be created and destroyed in the lab would apply just as much to these permitted practices.

Researchers at Rosling however believe that therapeutic cloning could be done without destroying human eggs or embryos. They are trying to create stem cells directly by adding the nucleus of adult cells to denucleated embryonic stem cells. This would remove many of the ethical objections to cloning-though not all of them. Researchers would still need to use stem cells derived from embryos. Such cells, however are now growing in the lab, so few, if any embryos would need to be destroyed to obtain more of them.

A new generation of vaccines is currently being developed with gene technology. Traditional vaccines are based either on a weakened or killed viruses of the disease agent, whereas the new vaccine uses only DNA coding for a particular segment of the disease causing organism.

DNA vaccination promises to be safer then previous vaccines, because it eliminates risks associated with contaminated vaccines. The immune response will be more long-lived and vaccines for various diseases can be given in a single shot. The new method will also be more cost-effective, since the vaccine development and production process is simplified with gene technology.

Researchers are already working with DNA vaccine for a larger number of vaccines. A promising DNA vaccine candidate for malaria is being developed, as well as new vaccines for AIDS, herpes and tuberculosis. Infact. DNA vaccination is very likely to be the future of vaccination.

A herd of cloned pigs may one-day be supplying organs suitable for transplantation into people. This was heralded by the cloning of a litter of 5 pigglets by a British Company. Pigs are an attractive source for transplants because their organs are of a similar size to those of humans, but normally pig organs are rejected by the human immune system within an hour. Pig cells carry sugars on their surface that act as "red tags" to the human immune system.

Genetic engineers can create pig cells without these sugars by disrupting the genes that enclose the enymes that process them. However they haven't been able to turn those cells into whole animals. Nucleus transfer, the technique used to produced Dolly, was a promising approach, but making the technology work in pigs turned out to be problematic. Sows require several viable embryos in a litter to carry a pregnancy to term, hence a different approach was used. Every step of the normal nuclease transfer had to be modified. Even so, the cloning efficiency was so low that "bucket loads of embryos" were put into the surrogate sow to produce 5 female clones.

In the field of agriculture, transgenic plants may be created with the aim of improving yield, prolonging shelf life, imparting disease resistance and improving nutritional value. The absence of studies concerning the long term effects of such manipulations and the possiblity of trigger genes being present and activated has been explored, this has led to calls for labeling of genetically engineered foods to outright bans.

Transgenic animals raise numerous ethical questions. It can be applied to the field of cancer research, the production of recombinant proteins may lead to unlimited sources of human insulin, human growth hormone, interferons etc. This raises the question of creating "superhumans", for now that does not seem possible.

This is but a small glimpse of what gene cloning might produce in the future. For now, though we know that Hitler is not coming back to life...

> *"Let me admonish you, first of all, to go alone; to refuse the good models, even those sacred in the imagination of men... Imitation cannot go above its model. The initiator dooms himself to mediocrity. The inventor did it because it was natural to him, it has a charm. In the imitator, Something else is natural, and he bereaves himself of his own beauty to come short or another man's"*
>
> *- Ralph Walso Emerson*

***Editor's Note***

*The authors are members of the student body - GENETICA, based in Alexander Brown Hall, UCH, Ibadan. Genetica is an educative club whose purpose, is to bring to light the importance of genetics, the recent developments in the field of genetics and to make this information available to medical students and the medical community as a whole.*