MOLECULAR CLONING
THE MANIPULATION OF THE GENE

—

From Sara El Gebali

PREFACE

To many contemporary areas in modern biology and medicine the methods to molecular cloning turned out to be central. Many people connect the word “cloning” with science fiction, even if the cloning actually is possible. Great quantities of small organisms are able to clone themselves to form whole colonies. The process in action is quite interesting by itself, but how far can we go with the approach that science already has discovered? And will humanity be able to clone anything, even human beings? To bring light on these questions, we have to understand molecular cloning and its procedures to a certain level.

INTRODUCTION

Context and recent events

In the area of biotechnology, molecular cloning exploits the fact of the fundamental structure of the DNA being the same in every biological organism. Presuming a piece of DNA is put together with the molecular arrangements needed for DNA replication, the developed recombinant DNA will have the unfamiliar DNA repeated in the host cell’s DNA of the transgenic living being.

The polymerase chain response (PCR) is quite similar. Both allow the replication of DNA arrangements. The difference is the molecular cloning incorporating the replication of the DNA in living organisms, while the PCR uses an in vitro arrangement, meaning it works outside of the main organism.[1]

This PCR procedure is the most used tool to help us identify a COVID-19 infection. It is similar, because with several steps the DNA segment is separated and duplicated, thus amplifying the chosen piece. A cycle repeats this process 30 to 40 times, making at least a billion copies in just a couple of hours. [2]

Molecular cloning progressed from cloning a single piece of DNA to connecting multiple sections of DNA to one single piece.

Recent scientific history

Earlier than the 1970s, the proper comprehension of genetics and molecular biology was inhibited due to the inability to separate and study sequences of genes of complex organisms. The approach to the molecular cloning methods, the technology changed fundamentally. Microbiologists were trying to understand the molecular function of a specific bacteria, which limits the growth of its bacteriophage. The enzyme called restriction endonuclease was isolated. Its function was to divide the DNA in a specific sequence. The second one was the DNA ligase, which joined the pieces to new combinations, the recombinant DNA. [1]

1. Cutting (Digestion)

The first recombinant DNA technology appeared in the late 1960s. The start was locating the enzyme responsible for slicing and joining the double strands. Even as early as 1952 a 'restriction factor' was discovered, limiting the growth of the bacteriophage. But the actual properties of the enzyme weren't discovered until 1968, when the isolation of the enzyme succeeded. The restriction factor cuts the exogenous part out, leaving the rest, because the methylase enzyme was protecting it.

After the discovery other scientists confirmed these studies by isolating the enzyme from the Influenza bacteria. Another characteristic of the enzyme was discovered: the ability to cut DNA in specific recognition sequences.

1. Assembling (Ligation)

The discovery of the ligase enzyme predated earlier observations. In the early 1960s scientists discovered the genetic combination following a breakage and a ligation. The linear DNA became multiple closed circles after an infection of the host. After just two years they were able to use it to assemble two fragments of DNA.

Shortly after the discovery of both enzymes, the first recombinant DNA molecule was fabricated.

2. Transformation

The new technology would be limited and molecular cloning impossible without being able to generate and isolate DNA. The incorporation and the expression of an unaccustomed genetic material was first seen, when a non-lethal strain of bacteria was altered into a lethal one.

The final experiment with all three to a recombinant DNA molecule was first accomplished in 1973, when the scientists were able to form a new strain of E.Coli by using these three steps, and thus laying the foundation of the recombinant DNA work. [3]

Application

Molecular cloning supplies scientists with a basically never ending quantity of any individual segments gained from any genome. The purposes for the obtained material have a big range, in both basic and applied biological science. Some important uses are summarized here.

Gene therapy

Gene therapy is providing a functional gene to cells, in which the function is absent. This could either correct a genetic disorder or an acquired disease. There are two major parts.

The adaptation of the germ cell leads to an everlasting genetic change in the whole organism and may cause change in future generations. This 'germ line gene therapy' is viewed as unethical to be used on humans.

The 'somatic cell gene therapy' aims at a specific tissue and treats it directly or removes it. If there is a removal, the tissue will be treated and then returned again. This therapy should help against cancers and disorders affecting the blood, the liver and the lungs.

Gene therapy has had limited success in history. A cause is the effect of the therapy only relieving a part of the disease, rather than curing them. The treatment itself also has consequences depending on the patient. Nonetheless it is a promising area in medicine and the research is active.

Transgenic organisms

The transgenic organism has a manipulated and characterized gene inserted in its DNA to show the targeted expression. Most of them were developed for basic research in biology, but others are used in the commercial range, such as crop plants with certain resistances and fluorescent fish for entertainment.[1]

Alternative treatments

CRISPR/Cas9 is currently undergoing research on how it could be used efficiently in the area of medicine in place of the traditional gene therapy. The clinical trials are still ongoing and there are still risks to that kind of treatment, but in sight to the future, it could exist as a better treatment than the gene therapy. A part of the hazards are mainly the ethical questions. If it would be possible to use CRISPR/Cas9 actively in the medical field, it would count as a revolution of sorts. [4][6]

ENGINEERING TECHNIQUES

Traditional cloning methods use recombinant DNA. The preparation of a vector starts to get the insert DNA with the restriction enzymes which splits the vectors. The fragemenst are spliced together by the ligase, forming a new vector. This is able to express the inserted gene. The method is simple and even old, but it's the groundwork for other methods, and may be even irreplaceable.

Vector preparations

The vectors are based on plasmids, which replicate on their own without interference of the genome. The vectors have a bacterial origin to extend more efficiently into the host cell. A marker, such as antibiotics resistance, is added. Commonly a multiple cloning site (MCS) is used, because it has several restriction enzyme sites, allowing faster addition of the wanted insert.

The restriction digestion will create an insertion site. Depending on the recognition sequence, different restriction enzymes could be used.

After, to stop self-ligation, a process of catalyzing a cut of a phosphate group from a phosphoprotein may be started. That process the phosphate group is removed at the 5' end. The ligation can't work, because there is a 5' phosphate and a 3' OH needed.

Insert preparation

The insert will have to undergo a restriction digestion, so the ends are compatible for the subsequent splicing into the vector.

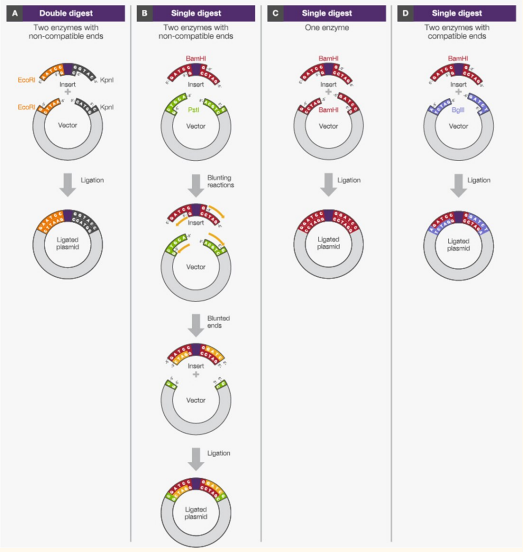


Figure 1. restriction enzyme cloning strategies

With a vector preparation, the suitable restriction enzymes for the cloning of the insert are chosen. A often used strategy is the process of the double digests on both the vector and insert. The picture below shows how the digestion works and how it could be fixed, depending if the ends are compatible or not.

In this process it's important for the reaction buffer and other conditions to be optimized for both enzymes, to avoid any mistakes.

After the restriction digestions of the vector and the insert, the desired fragments are polished by putting the samples into agarose gel and taking the piece of interest out. The gel electrophoresis is also great for removing the leftover enzymes and salts.

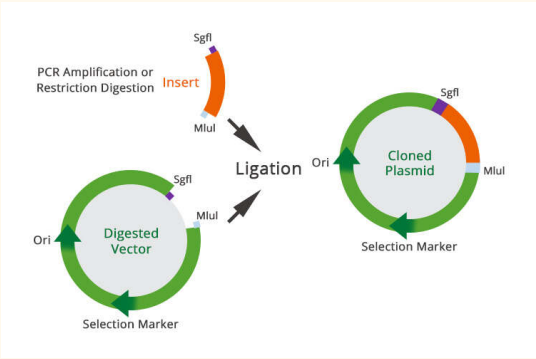


Figure 2. The ligation of a vector and an insert

Ligation

The moment the wanted piece is taken out, the ligation process can be started to join the insert and vector. The ligase is different depending on the lab and others. It could also need some other substances to really work, but also this is individual.

The temperature range lies between 14°C to 25°C with a reaction time from 10 minutes to almost 16 or even overnight. The exact numbers depend on the type of DNA and what the desired outcome is.

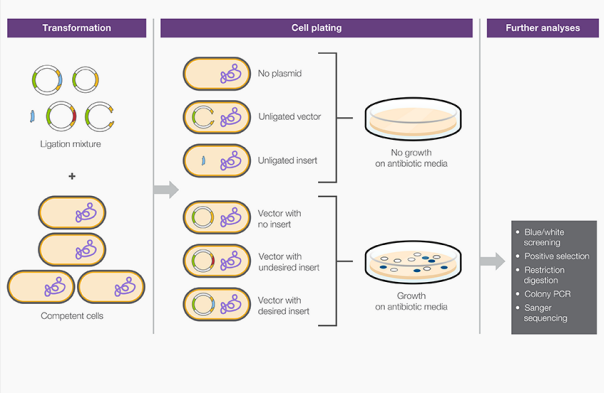
Transformation

This part of the process is natural. The bacterial cell accepts the foreign DNA at a low frequency. If the cell is made porous the process could be enhanced.

These competent cells are often treated with calcium chloride, making it the desired porous. Mixing that with a heat-shock at 42°C, the absorption of the DNA is happening easier. Another possibility is electroporation. The bacteria is treated with an electrical current, creating pores in the membrane.

Colony screening

After the transformation reaction there are multiple possible outcomes. The mix contains cells with no vector with no insert, the insert alone, and the successfully ligated vector and insert. The bacterial cells without the vector do not have the marker, which is the antibiotic resistance and thus will perish in these conditions.



The mixture could have failed products, like an unligated insert, unligated vector and empty vectors. There is a possibility of undesired inserts in the vectors. These may be incorrect, or have a mutated sequence.

A common method to identify the inserts are the “blue/white” screening and positive selection.

Figure 3. Mixture of bacteria after transformation and their phenotypes (growth/no growth/blue-white) on antibiotics selection media plate.

The positive selection is activating a gene lethal to the bacterial host located in the MCS of the vector. If the ligation of the insert was successful it wouldn’t be expressed, leading only the transformed cells to survive.

The presence of the insert can also be shown with a PCR. This method needs PCR primers specific to the insert and to the flanking vectors.

Once clones with the correct insert are named, they are ready for the next experiments.[5]

INTERVIEW

Q: My first question would be: what are you currently working on and how does it work?
A: At the moment we are looking at the process of transportation in a cell. You could imagine the cell as a warehouse. The products inside it have to be transported from A to B. We are basically looking at the different components being carried from one place to the other and how it will be used later on.

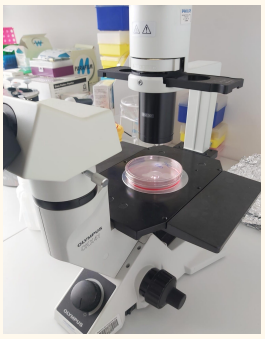
Q: How does that work in connection with molecular cloning?
A: Well, how is it even possible to understand the transportation process inside of the cell? It's simply not possible. That leads to us making artificial constructs, that's where the cloning takes place. We tinker, basically like Lego, with the DNA. With that, in addition to a model protein to have an overview of the process, we add an invented sequence, which could react later. That sequence is often specific to a place in the cell. With this modified cell we can discover where the reaction actually happens. This way we are able to follow up the transportation. That's where the cloning is happening on a DNA level, rather than cloning a whole organism.

Q: Is molecular cloning developed to actually use DNA as building blocks?
A: Yes, after the discovery of restriction enzymes it got a lot easier. We are actively able to put DNA together as if they're blocks of lego. But something newer is CRISPR/Cas.

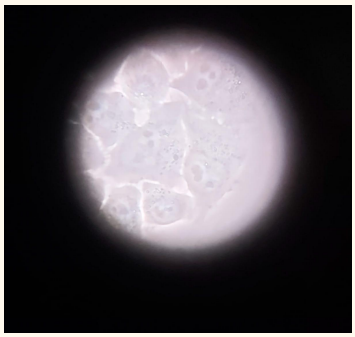
Q: Okay, but connecting to that, what are the recent events? Will they change anything in the area of research?
A: Basically, we're not using molecular cloning on humans, but we are using it to discover interesting processes to understand those more. There are a lot of new DNA sequences, which help us with our doing. Like the markers, making the wanted DNA fluorescent etc.. We do have a lot of new discoveries like these. But the main idea of molecular cloning will stay the same. It may look different from the outside, with new tools, but the baseline is still the same.

Q: On another subject, how is CRISPR/Cas currently used in that field? Is it only useful for proteins, or can it be used as a substitute for gene therapy?
A: Good question, gene therapy is still not accepted in our society. CRISPR/Cas would be ideal, because it could help against the point mutations, which are the main reason for a lot of illness. We could basically redesign the gene. That's why it would be great as a gene therapy. But it's still ethically questionable. On the biological and medical line, it surely would be useful. But sadly the research on the CRISPR/Cas is limited because of the ethical boundaries set on us. It's just not accepted in society. Given the possibility, it would be the best gene therapy.

Q: You talked about the point mutation. Would CRISPR/Cas be able to efficiently cut that mutation out? And would the age/development make a difference?



A: It really depends. Every cell has the same genome. But there is a possibility of the mutation being only expressed in one kind of cell. Then you would only remove it from that kind of cell. But you would have to replace every single cell with that technique. Which is just not possible. But depending on the mutation, you could make a virus, which would fix that mutation with CRIPSR/Cas9. It's a local repair and that would be in fact possible. But every cell? That wouldn't work.



Q: Does the CRISPR/Cas have a specific role or is it universally usable?

A: The function of the Cas is the Screening. For that it needs a guide. The moment both are connected, you can send it to look for the wanted gene. And the moment it discovers the complementary gene, it will cut there. Cas is basically just an enzyme. It's not something incredible that can't be replaced. It is necessary, but there are other enzymes, which could also be used. [6]

Figure 4 & 6. human cells manipulated and cloned to produce a specific protein.

DISCUSSION

Progress

The application of the chosen technique will never truly change. The process and how the cloning happens will always stay the same. It may change with new tools, but the main idea will remain the same. As for the usage of molecular cloning in society, it also will stay the same. It will be a tool to discover processes and hidden features in a gene. Maybe there will be some progress with it in the medical field, but due to the ethical difficulties, it will have a long way in front of it.[6]

Future steps

The future steps will be probably with CRISPR/Cas, as they offer even some gene manipulation. The ability of cloning a cell and maybe replacing nonfunctional ones will be worked on to help with several illnesses in the future. With molecular cloning the discovery of new means to battle genetic dysfunction is a possibility. Insulin is a great example, how cloning affects biopharmaceuticals.

Discussion of ethical aspects

The discussion of the ethical aspects started in 1966. At first it was looked at from a religious point of view. Ramsey P. a Protestant theologian condemned cloning, as it was against the christian beliefs. In his opinion, the possibility of cloning could change the way people view human happiness, morality, personhood, power and procreation.

Another theologian, Fletcher J. argued for the vast possibilities of cloning. He mentioned being able to control the offspring with these methods. His argument is based on the fact of humans wanting to maximize their well-being. And a customized child is better than any other one. His arguments lead to the "new" eugenics, which specializes in the fetal genetic manipulation and aims to identify and eliminate 'defect' genetic material. [7]

Even in 1977 the whole cloning ordeal wasn't ethically justifiable. The University of Maryland published a report, claiming:

" […] at this time, it is morally unacceptable for anyone in the public or private sector, whether in a research or clinical setting, to attempt to create a child using somatic cell nuclear transfer cloning. […]" (Report from the Institute of Philosophy and Public Policy, 1997, p. 2)

This statement resulted from the uncertainty a cloning would bring forth, such as mutations or health complications. This leads to the assumption that the ethical problem is more likely going to be the success and the following consequences, rather than the process in itself.

Other concerns raised in the report are the being of the clone itself. How is it possible for a clone to retain a sense of self, if it is 'only' a copy? Even if the clone would be rather a twin, than an exact copy of a person, due the nurturing factor still playing a role. [8]

Most of these arguments still play a role, since the human has a more complex structure of genes than maybe plants.

Opportunities & Threats

The opportunities of cloning are vast. As an example, it could be used to regrow whole organs and tissues. This way doctors could perform surgery without having to worry about an organ donor. Not to mention the studies the researchers could perform on cloned stem cells, to discover new processes and proteins.

There is also a possibility of bringing back extinct animals with only their DNA. The ability to see long lost species would help research.[9]

Which leads to the opportunity to synthesize meat cheaper and without any animal cruelty. Not only would the poor people profit from this, but also the animals.

But on the other hand, if there is a possibility to clone actual humans, the system would be unpredictable. Not only would eugenics play a bigger role in this, but the genetic variation would also decrease. This comes from the people modifying the 'perfect' children. This could result in the possibility of disease and deformity being higher and requiring more genetic selection to fix that again.
Also the rising of eugenics could lead to socioeconomic problems, as the rich will be the only really able to make the clones, making the gap between rich and poor bigger. [10]

SUMMARY

In conclusion, molecular cloning is a rather new tool. Even so, the main procedures will not change, because these are essentially the fundamentals. The techniques require a lot of patience and handiwork and precision. At the moment it is used to discover how simple proceedings work in a cell, but maybe the tools of this could get so advanced to actually help some illnesses. This would only work if the difficulties are overcome and if the ethical part has an official consensus on that topic. Especially with the uncertainties that lay in this topic. Once this is achieved, molecular cloning could help many people in the medical field. There are a lot of strengths but also a lot of weaknesses, which will result in an imbalance in our society. But maybe the opportunities could outweigh the threats, if there is a fitting law.

Sources

1. *Molecular cloning,* 17 January 2022, Wikipedia, accessed 12 February 2022, <https://en.wikipedia.org/wiki/Molecular_cloning >
2. *Polymerase Chain Reaction (PCR) Fact Sheet,* 17 August 2020, National Human Genome Research Institute, accessed 12 February 2022, <https://www.genome.gov/about-genomics/fact-sheets/Polymerase-Chain-Reaction-Fact-Sheet >
3. Rebecca Tirabassi, Bitesize Bio, u. d., *Foundations of Molecular Cloning - Past, Present and Future,* New England Biolabs, accessed 12 February 2022, <https://international.neb.com/tools-and-resources/feature-articles/foundations-of-molecular-cloning-past-present-and-future >
4. Fathema Uddin/ Charles M. Rudin/Triparna Sen, 07 August 2020, *CRISPR Gene Therapy: Applications, Limitations, and Implications for the Future,* frontiers, accessed 12 February 2022, < https://www.frontiersin.org/articles/10.3389/fonc.2020.01387/full >
5. *Traditional Cloning Basics*, u. d., ThermoFisher Scientific, accessed 12 February 2022, <https://www.thermofisher.com/ch/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/molecular-cloning/cloning/traditional-cloning-basics.html >
6. Dominik Buser, PhD, Lecturer and Scientist for Molecular Biology, Biozentrum, University of Basel
7. Matti Häyry, Ethics and cloning, *British Medical Bulletin*, Volume 128, Issue 1, December 2018, Pages 15–21, accessed 12 February, <https://academic.oup.com/bmb/article/128/1/15/5094025#131999628 >
8. R. Wachbroit, Fall 1997, *Genetic Encores: The Ethics of Human Cloning,* Philosophy & Public Policy, Volume 17, Number 4, ISSN 1067-2478, accessed 12 February 2022, <http://ojs2.gmu.edu/PPPQ/article/viewFile/278/206 >
9. Laurie Brenner, 27 July 2018, *Advantages & Disadvantages of Cloning*, Sciencing, accessed 12 February 2022, <https://sciencing.com/advantages-disadvantages-cloning-4620621.html >

10. Louise Gaille, 4 October 2017, *11 Advantages and Disadvantages of Cloning,* Vittana, accessed 12 February 2022, <https://vittana.org/11-advantages-and-disadvantages-of-cloning >

Figure 1: *Traditional Cloning Basics*, u. d., ThermoFisher Scientific, accessed 12 February 2022, <https://www.thermofisher.com/ch/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/molecular-cloning/cloning/traditional-cloning-basics.html >

Figure 2: *Tips on Molecular Cloning,* 12 March 2019, Origene, accessed 12 February 2022, <https://www.origene.com/blog/molecular-cloning >

Figure 3: *Traditional Cloning Basics*, u. d., ThermoFisher Scientific, accessed 12 February 2022, <https://www.thermofisher.com/ch/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/molecular-cloning/cloning/traditional-cloning-basics.html >

Figure 4 & 5: Dominik Buser, PhD, Lecturer and Scientist for Molecular Biology, Biozentrum, University of Basel