Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Treatment of 27 Different Types of Cancer

Mihai Bojescu (misubojescu@gmail.com)
Collège et École de Commerce Émilie-Gourd Switzerland

Research

Keywords: cancer , VP1 protein, REP genes , rAAV

Posted Date: March 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-249275/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Abstract

Background

This research paper analyzes how to treat cancer with a virus. It is known since the 1960s that viruses could be used to fight against cancers.

Methods

To answer this question, we analyzed the receptors that mutates when the genetic code becomes corrupt, then we analyzed the standard VP1 protein of two viruses of the same family. After that, we combined these two elements. Then we studied the REP genes and their impact on the virus entrance and replication. Finally, we examined the protocol of the synthesis of a rAAV.

Results

Our results showed that the protocol is applicable and that we would obtain a virus specially designed to kill the cancer cells. This virus would have a fully operational DNA, which would contain the VP1/2/3 genomic code, the REP genes and the ITR regions.

Conclusion

This research is only theoretical, and all the steps and methods are based on a strong theoretic basis. The experimentation has not been made. We did not have the material to test them, but we tried to take all the impacting factors in consideration. Our study has shown that a treatment against cancer consisting of a virus could be possible.

Importance

This research is extremely important. It could allow the eradication of cancer from earth. It is a major step in oncogenesis, and it could provide a large amount of fame to the journal that would publish it.

Introduction

To this day, due to numerous environmental and genetical factors, the number of new cancer cases increases, demanding a new way to treat this disease.

The propriety of oncolytic viruses has been realized in the 60s and the first vectors have been used in the early 2000. The problem was that these viruses were only useful for specified tissues in specific organ. This new theoretical treatment is important because it can treat 27 different cancers with the adenovirus.

Although the researches made in the mid-20 century were valuable, the technology to create synthetic viruses was not developed yet, which conducted to the research of natural oncolytic viruses. This approach was not concluded with success.

We conducted this research with the scope to find a specific protein that mutates when a genetic mutation occurs. This contact protein is the atypical chemokine receptor 3. By using this mutated receptor, a virus could enter in the cancer cells only. The most positive point is that 27 different tissues have this receptor. These organs are:

1. The Thyroid gland
2. The lungs
3. The salivary gland
4. The Esophagus
5. The stomach
6. The Duodenum
7. The small Intestine
8. The Colon
9. The Rectum
10. The Liver
11. The Gallbladder
12. The Pancreas
13. The Kidney
14. The Urinary Bladder
15. The Testis
16. The Epididymis
17. The Fallopian tube
18. The Endometrium
19. The Breast

20. The Hearth
21. The skeletal
22. The Adipose tissue
23. The Soft tissue
24. The skin
25. The appendix
26. The lymph node
27. The tonsil

Methods

The research conducted by NCBI, has proved that a new way of protein expression was possible. I used their way of protein expression. This method consist of ten steps [7].

1. Augmentation of Plasmids Containing Required Coding (see Fig. 1) DNA Sequences (CDS) (coding DNA sequences)

We pre-warm the SOC (Super Optimal Broth) medium to room temperature and the LB (Luria broth) agar plates containing 100 µg/ml ampicillin to 37°C. Then, we equilibrate the water bath to 42°C.

We thaw one vial of chemically component E. coli on ice.

After that, we add 1–5 µl of the plasmid (10 pg to 100 ng) containing the CDS into the vial of chemically component E. coli and we mix gently. After the adding of plasmids, we mix by tapping the tube gently. After that, we incubated the mixture on ice for 30 min. Then, we heat-shock the E. coli for 30 sec at 42°C in a water bath without shaking and place the vial on ice for 2 min. Then, we add 250 µl of pre-warmed SOC medium to the E. coli and shack the bacteria horizontally at 300 rpm for 1 hr. at 37°C in a bacterial incubator. After that, we spread 100 µl and 150 µl from transformation mix on pre-warm LB agar plates and invert the plates and incubate overnight at 37°C. After colonies become visible, we inoculate a single colony from each plate into a 15 ml culture tube containing 5 ml LB medium with 100 µg/ml ampicillin. Then, we incubate them overnight at 37°C with shaking at 300 rpm until culture is in late log or stationary phase. Then, we isolate the plasmids containing the required CDS and we determine the plasmid concentration using a spectrophotometer. We also prepare aliquots of 20 µl and store them at -20°C for a prolonged time.

2. Amplification of Plasmid Inserts and Adding of Poly T-tail (Poly thymine tail) by Polymerase Chain Reaction (PCR)

To obtain the DNA template for the in vitro transcription, (IVT) (in vitro transcription) eGFP (Green fluorescent protein) is amplified using PCR (Polymerase Chain Reaction). Simultaneously, a poly T-tail of 120 thymidines (T) is added to the insert by using a reverse primer with a T_{120} extension. Thereby, the generated mRNAs obtain a poly A-tail with a defined length after the IVT. Then, we prepare the PCR mixture and we mix the reaction. After that, we place the PCR tubes in a thermocycler and run the PCR using the PCR cycling protocol. We also clean up the PCR reaction using a PCR purification kit according to manufacturer's instructions and elute the DNA using 20 µl nuclease-free water. We also measure the concentration of the DNA using a spectrophotometer. Finally, we freeze the DNA at -20°C for a long time or use it directly for IVT.

3. In Vitro Transcription (IVT)

After PCR, the plasmid inserts are amplified and a poly T-tail is added. Then, we prepare the NTP/cap (Nucleosidtriphosphate/cap) analog mixture as described in. We also mix the NTP/cap analog mixture thoroughly by vortexing and spin down briefly. After that, we mix the IVT reaction mixture thoroughly by gently pipetting up and down. Then, we centrifuge the PCR tube briefly to collect the mixture at the bottom of the tube. We also incubate at 37°C for 3 hr. in a thermomixer. To remove the template DNA, we add 1 µl of DNase (2 U/µl) to the IVT reaction mixture. Then, we mix well and incubate for 15 min at 37°C. After this process, we purify the reaction mixture using a RNA purification kit. We also elute the modified mRNA from the spin column membrane twice with 40 µl nuclease-free water.

4. Treatment of Purified mRNA with Antarctic Phosphatase

We add 9 µl of 10x Antarctic phosphatase reaction buffer to 79 µl of purified mRNA solution. Subsequently, 2 µl of Antarctic phosphatase is added (5 U/µl) and the sample is gently mixed. The reaction mixture is incubated at 37°C for 30 min.

Then, we purify the reaction mixture using an RNA purification kit. We also elute the modified mRNA from the spin column membrane twice with 50 µl nuclease-free water.

After that, we measure the concentration of the modified mRNA using a spectrophotometer. We also check that the ratio of absorbance at 260 nm/280. The modified mRNA is aliquoted into single-use aliquots required for transfections and they are stored at -80°C.

5. Preparing of Cells for Transfection

We plate 2 x10^5 cells (HEK293 cells) per well of 12-well plate and we incubate the cells overnight at 37°C in a cell incubator.

6. Performing mRNA Transfection of Cells

We thaw the modified mRNA and we generate the lipoplexes for transfection. 25 µl (2.5 µg) of modified mRNA and 2 µl of cationic lipid transfection reagent to 473 µl Opti-MEM (Minimal Essential Medium) I reduced serum medium is added. We scale up the volumes according to the number of wells to be transfected. We also mix the components gently by pipetting. The transfection mixture is incubated at room temperature for 20 min. The cells are washed with 500 µl DPBS (Dulbecco's phosphate-buffered saline)/well and we add 500 µl transfection mixture to one well of a 12-well plate. The cells are incubated for 4 hr at 37°C and 5% CO_2. Then, we aspirate the transfection mixture and we add 1 ml complete cell culture medium to the cells. They are incubated for 24 hr in the cell incubator.

This is the obtained mRNA code;

Atggctgctgacggttatcttccagattggctcgaggacaacctttctgaaggcattcgtgagtggtgggctctgaaacctggagtccctcaacccaaagcgaaccaacaacaccaggacaaccgtcggggtcttgtgcttc

Manufacturing of CAP genes

Conform the research conducted by NCBI, three steps are evidenced: Cloning of VP3, Construction of VP1/VP3 and Assembly of VP2

Cloning of VP3.

A synthetic gene encoding VP3, which are residues 735 to 972 of the IPNV (infectious pancreatic necrosis virus) strain Jasper structural polyprotein, with a DNA sequence optimized for Escherichia coli expression but an unchanged protein sequence is amplified by PCR using KOD DNA polymerase (forward primer, 5'-AAGTTCTGTTTCAGGGCCCGACCGCAAGCGGTATGGATGCAG-3'; reverse primer, 5'-ATGGTCTAGAAAGCTTTAAACTTCACCATCATCACCGCTCG-3'). The PCR product is cloned into the pOPINF expression vector using ligation-independent [8].

[see Fig. 2] Construction of VP1/VP3

VP3 is expressed in E. coli B834 cells grown in the presence of 50 µg/ml carbenicillin. The cultures are also grown at 37°C until the optical density at 600 nm reached 0.6, at which point they are cooled to 20°C and the protein expression is induced by adding 0.5 mM isopropyl-β-d-thiogalactopyranoside. The cells are incubated for a further 20 h, harvested by centrifugation at 8,000 × g, 8°C, 20 min, and they are stored frozen at − 80°C. After that, the cell pellets are thawed and resuspended on ice in 50 mM Tris (trisaminomethane), 500 mM NaCl, 50 mM imidazole, and 0.2% (vol/vol) Tween 20 with a pH of 7.5, supplemented with 400 units of bovine pancreas DNase I and with one EDTA-free protease inhibitor cocktail tablet (1 liter). The cells are lysed using either at 30,000 lb/in^2, and the cell lysates are cleared by centrifugation at 35,000 × g, 8°C in 30 min. The cleared lysate is applied to a 1-ml HisTrap Ni^{2+} affinity column, which is washed with 50 mM Tris, 500 mM NaCl, and 50 mM imidazole at pH of 7.5, and eluted in 50 mM Tris, 500 mM NaCl, and 500 mM imidazole, also at pH 7.5. The eluate is further purified by size exclusion chromatography using the HiLoad 16/60 Superdex at 200 columns equilibrated in 20 mM Tris and 200 mM NaCl of a pH of 7.5 and an ÄKTA Purifier 10 UPC. The fractions containing the purified VP3 are pooled and incubated with 50 µl of rhinovirus 3C protease. We start with a concentration of 2 mg/ml. Then, we wait overnight at 4°C, to remove the N-terminal His_6 tag before they are being passed through a 1-ml of HisTrap Ni^{2+} affinity. Once purified, the untagged VP3 at 1 mg/ml is mixed with either full-length and incubated for 1 h at 4°C to facilitate the formation of the complex. The VP1-VP3 complex is also purified by a Ni^{2+} affinity chromatography, followed by a size exclusion chromatography. Finally, the fractions containing the pure complex are pooled and concentrated in 30,000-molecular-weight-cutoff centrifugal concentrators.

UAC CGA CGA CUG CCA AUA GAA GGU CUA ACC GAG CUC CUG UUG GAA AGA CUU CCG UAA_ GCA CUC ACC ACC CGA GAC UUU GGA CCU CAG GGA GUU GGG UUU CGC UUG GUU GUU GUG GUC CUG UUG GCA GCC CCA GAA CAC GAA GGC CCA AUG UUU AUG GAG CCU GGG CCA UUG CCU GAG CUG UUU CCU CUC GGC CAG UUG CUC CGC CUG CGC CGU CGG GAG CUU GUG CUG UUU CGA AUG CUG GUC GUC GAG UUC CGG CCA CUG UUG GGC AUG GAG UUC AUG UUG GUG CGG CUG CGG CUC AAA GUC CUC GCA GAA GUU CUU CUA UGC AGA AAA CCC CCG UUG GAA CCG UCU CGU CAG AAG GUC CGG UUU UUC UCC UAG_ GAU ACC UAG_ ACG UAG_ ACA AAC UAA_ UAU CGC UUG GCC CGU UGA_ AAU CGC UAU AAU CGA CCG GCA CGU UGU CGU CGC UAA_ CGU AAC ACC ACC UAU GGC ACU ACA CGG GCU UGU ACG GCU UGU UUU CGC ACG ACG ACA UAU GGG ACU CGA AAU AAA UAU AAA AAU AAA AAC ACU AAC CGU ACU AAC GCU UGU CGC ACC ACC ACA CCC ACU UGU AAG UCC GCU UUU GGU GGC CGA UAC UAU GGG UAA_ CGA UAU AAG ACU UGG ACC GCU AAC GCC UAG_ ACA CCC ACC ACG ACU GGU AAG GCC ACA CCC ACC ACU CGG ACC ACG UCG UAU UGG UCA CCG GCU ACC CGC UUG ACU GGA CGU UUC ACU GGG UAG_ ACU AAA AAU CGU AAU UGG ACA AAC CGU CGU AAA AAA AAG ACU GGA CGU ACU CGC ACC UAG_ CGA UAG_ ACU CGU AAU GGA UAA_ AAU GGU UGU GGG GCU CGU CGG CGU UUU UUU ACC ACG CGG CGC ACC ACA CGU AAG ACC ACA CCG ACG ACC GCA AAA CGC ACU CGG ACG GCC UAU GGA UAA_ UAG_ ACU UUU GGC ACU GGU CGC GCU CGU UGU UGC UUU GGA UAA_ CGG CGU CGA AAA UAG_ GCC UUG UAU CGU AAU UUC UUA CCG ACU AAC CGU ACC UUG ACC ACU CGC ACC ACG ACC CGA AAC GCC ACG GCA AAU CGU AAU AAC GCC ACA AAA UAA_ AAG ACG ACC GCG CGC GCU AAU CGC GCU CGU CGC UAG_ UCC UUU UUG UAU CGU CGG CGU UUU AAU AAA AAU CGA UAC ACC ACC ACA AAG ACC ACA CGA CCG ACG GCA UAG_ UAC ACC GCC ACG ACG ACC UAU AAA AAU CGU AAG ACG UAA_ UAU AAG GCA AAU GGA CGG CGG ACC UUG UAC GCG ACA AAU GGC GCG ACG UAC ACU GGC GUC GGG GGU GUU CAA ACC CUA GAU UAU GUU ACC GAA GUC CGC CAC CGC GUG GUU ACC GUC UGU UAU UGC UCC CAC GGC UAC CUC ACC CAU UAA_ GGA GUC CUU UAA_ CCG UAA_ CGC UAA_ GGG UUA CCG ACC CGC UGU CUC AGU AGU GGU GGU CGU GGU CUU GGA CCC GGG ACG GGU GAA UGU UGU UGG UAG_ AGA UGU UCG UUU AGA GGU CGG UUA GUC CUC GAA GUU UGC UGU UGG UGA_ UGA_ AAC CGA UGU CGU GGG GAA CCC CCA UAA_ AAC UGA_ AAU UGU CUA AGG UGA_ CGG UGA_ AGA GUG GUG CAC UGA_ CCG UCG CUG AGU AAU UGU UGU UGA_ CCC CUA AGG CCG GGU UCU UUG AGU CGA AGU UCG AGA AGU UGU AGG UUC AAU CUC CCC AGU GCG UCU UGC UAC CGU GCU GCU GAU AAC GGU UAU UGG AAU GGU CGU GCC AAG UUC ACA AAU GCC UGA_ GCC UCA UAG_ UCG AGG GCA UGC ACG AGC CCA GCC GCG

UGG UUC CGA CAG AGG GCG GCA AAG GUC GCC UGC AGA AGU ACC AGG GAG UCA UAC CUA UGG AGU GGG ACU UGU UGC CUU CAG UUC GCC ACC CUG CGA GUA GGA AAA UGA_ CGG ACC UCA UGA_ AGG GAA GCG UCU ACG AUU CCU GAC CUU UAU UGA_ AGG UUA AGU CGA UAU GGA AGC UCC UAC AUG GAA AAG UGU CGU CGA UGC GAG UGU CGG UCU CAA ACC UAG_ CGA ACU ACU UAG_ GAG AAU AAC UAG_ UCA UAG_ ACA UGA_ UGG ACU UGU CUU GCG UUC CUU GUU GGA GAC CUU GUU GGU UGG UUA GUG CCG ACG AAA AAU CGG UCC GAC CCG GAG UCA GAU ACA GAA ACG UCC GGU CUU UAA_ CCG AUG GAC CCG GGA CGA UGG CCG UUG UCU CUG AAA GUU UCU GAC GAU UGC UGU UGU UGU UGU CAU UGA_ AAG GAA CCU GUC GCC GGU CGU UUA UAG_ UAG_ AGU UAC CGG CGC UGA_ GCG ACC ACU UAG_ GUC CUG GUC GAU ACC GGU CAG UGU UCC UGC UAC UUC UUU UUA AAA AGG GAU ACG UGC CGU UAG_ AUU AUA AAC CGU UUC UUC CCU GUU GCC GUU CAU UGC GUC UUA AUC UAU UAC AUU ACU AAU GCC UAC UUC UUC UCU AAG CAU GGU GGU UAG_ GAC ACC GUU GUC UCG UCA UAC CUU GAC ACC GUU UAU UGA_ ACG UCU CGA GUU UAU GUC GAG GGU GCU GAC CUU GAC AGU UAG_ UAG_ UCC CCC GGA AUG GAC CGU ACC ACA CCG UUC UAG_ CAC UGC ACA UGG AAG UUC CUG GAU AGA CCC GUU UCU AAG GAG UGU GCC UAC CUG UGA_ AAG UAG_ GAA GAG GAG ACU ACC CUC CGA AAC CUG ACU UUG UAG_ GCG GAG GAG UUU AGU ACU AGU UUU UAU GAG GCC AUG GCC GUU UAG_ GAG GCU GCU GAA AGU CGG GCC GGU UCA AAC GAA GUA AAU AGU GAG UCA UGA_ GGU GAC CUG UCC AGU CGC ACC UUU AAC UCA CCC UCG AUG UCU UUC UUU UGU CGU UUG CAA CCU UAG_ GUC UCU AAG UCA UGU GAA GGU UGA_ UGU UGU UCA GAC AAU UAC ACC UGA_ AAU GAC AUC UGU GAU UAC CAC AAA UAU CAC UUG GAG CGG GAU AAC CUU GGG CCA UAG_ AGU GUG CUU UGA_ ACA CU

Assembly of VP1/VP3 with the mRNA.

These are the steps to assembly the mRNA and the VP1/VP3 genes.

The IPNV RdRP (infectious pancreatic necrosis RNA-dependent RNA polymerase) is expressed and purified, and the $s\Delta^{+}_{CCC}$ single-stranded RNA template is also generated (see Fig. 2). The VP1-catalyzed replication assays are incubated with 50-fold molar excess of the full-length VP3 protein for 10 min on ice prior to the addition of the ssRNA template and nucleotides. We also add 1 mM ATP (Adenosine triphosphate), GTP (Guanosine-5'-triphosphate), and 0.2 mM CTP (Cytidine triphosphate), UTP (Uridine-5'-triphosphate) supplemented with 0.3 pmol (picomole) [α^{32}P]-UTP. After 2 h of incubation at 37°C, the reactions are stopped by the addition of 2× loading buffer [8].

Assembly of VP2[8] These are the steps to create VP2;

1.1. Construction of recombinant plasmids and recombinant bacmid

The Genomic DNA is extracted from a cell-cultured strain 20 – 06 of PPV (porcine parvovirus) by the phenol-chloroform extraction method. It is used as a template to amplify the VP2 fragment by Polymerase Chain Reaction. The PPV-VP2 gene is amplified with the primers PPV-VP2 FD (porcine parvovirus VP2 protein), containing TATGGATCCGATGAGTCATCATCACCATCACCATAGTGAAAATGTGGAACAAC and PPV-VP2 RV, GCGTCGACTATGAGTTAGAGTTTGTATTAG. The PCR products must be digested with BamHI and SalI and subsequently cloned into the corresponding restriction sites of the pFastbac1 vector to produce the recombinant plasmid, pFastPVP2. The insert of the recombinant plasmid is confirmed by DNA sequencing.

After the recombinant pFastPVP2 giver plasmid is resolved to be right, the DNA is changed into a DH10Bac™. The white colonies contain the recombinant bacmid, and, therefore, they are chosen for isolation of the recombinant bacmid DNA. Before the DNA is separated, up-and-comer colonies were streaked to guarantee they were genuinely white. Bacmid DNA (B-pFastPVP2) is extricated by the phenol-chloroform extraction strategy. The recombinant Bacmid (B-pFastPVP2) is then examined by PCR.

1.2. Expression of the VP2 protein in sf9 (Spodoptera frugiperda 9) cells

The recombinant baculoviruses, containing the coding sequences of VP2 with the polyhistidine tag at the N-terminus, are generated via the use of the Bac-to-BacTM system. Propagation of the recombinant virus is carried out in accordance to standard procedures. For manufacturing of the recombinant VP2 proteins, sf9 cells had been grown in 2-l Erlenmeyer flasks on orbital shakers at 120 rpm to a concentration of about two × 106 cells per ml of culture medium, in an 30 ml growing volume and contaminated with the recombinant viruses at a multiplicity of infection of 2–3. At seventy-two h post infection, cells are collected and processed. The procedure is as follow : The contaminated cells are collected via low-speed centrifugation at 3500 × g swing-out rotor four × 750 ml for 15 min at 4°C and solubilized in 30 ml of ice-cold lysis buffer, with 20 mM Tris, 0.3 M NaCl, 1.0% (v/v) Triton X-100, at a pH of 7.4 for 15 min with mild mixing. The crude cell lysate is clarified through high-speed centrifugation at 23,400 × g for 20 min at 4°C. The supernatant fraction is collected, and the soluble recombinant protein products purified via IMAC (Immobilized metal affinity chromatography).

1.3. Purification of PPV VP2 protein

Cells are harvested at distinct times after infection, centrifuged at 200 × g for 15 min, and resuspended in 25 mM Na2HCO3 with a pH of 8.3, at a density of two × 107 cells/ml. The lysis can occur for 20 min. Afterwards, cell debris are eliminated by using centrifugation at 10,000 g for 15 min. The recombinant fusion of the VP2 protein is purified by means of IMAC. The clarified lysate is incubated with three ml of pre-equilibrated Ni2+- streamline shelating GelTM on a rotating wheel for sixteen hours at 4°C. Then, it is then placed in a 10-ml chromatography column. The weakly bound and contaminating proteins are washed from the chelating gel through the usage of 10× the column volume with 20 mM Tris, 0.3 M NaCl and 20 mM imidazole, at a pH of 7.4. The recombinant polyhistidine-tagged protein products are ultimately eluted from the packed bed with 3–4× the column volume, with 20 mM Tris, 0.3 MNaCl and five hundred mM Mimidazole, at a pH of 7.4. One-ml fractions are collected.

1.4. SDS (Sodium Dodecyl Sulfate)-PAGE and Western blotting

The purity and the apparent molecular weight of the recombinant VP2 proteins are assessed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analysis. The purified proteins are separated by using the SDS-PAGE and are stained by transferring onto nitrocellulose membranes using a moist transfer cell for Western blotting. The protein expressed zero to 5 days after insect cells are challenged is obtained for Western blotting. The membranes are blocked with 5% skimmed milk in TBS-T, with 50 mM Tris-HCl, one hundred fifty mM NaCl; 0.05% Tween 20, at a pH of 7.5 for 1 h at room temperature. The Swine anti-PPV (anti porcine parvovirus) sera, with a 1:1000 is introduced to the membranes and shaken overnight at 4°C.

Manufacturing of Rep genes

Conform the research conducted by the journal of Virology, the REP genes can be simply created. I used their seven steps because the research conducted by ABM demonstrated that REP genes are not important in rAAV's process to kill cancer cells.

Recombinant plasmid DNAs.

For inducible expression of the AAV-2 Rep proteins an expression assembly primarily based on the MMTV-LTR (Mouse mammary tumor virus long terminal repeat) is generated. The central HaeIII subfragment of the MMTV-LTR is subcloned in pBluescript collectively with a chicken P-globin (platypus β-globin) polyadenylation signal. On the construct, a tkneo cassette is inserted, yielding plasmid pMtneo. The rep open analyzing body which includes the sequence for the C terminus of the spliced Rep proteins is inserted into pMtneo to yield pMtrep.

The expression constructs for rep4O, which is pCMrep40, rep52 which is pCMrep52, rep68 which is pCMrep68, and rep78 which is pCMrep78 are primarily based on the pKEX, which contains the human cytomegalovirus immediate early promoter. The separated expression of the individual Rep proteins is executed with the aid of mutating the inner translational start AUG, at the position of 993 for the synthesis of rep52 and rep40 proteins in plasmids pCMrep78 and pCMrep68, with the aid of mutating the splice donor site, which is G1907A in the case of pCMrep52 and pCMrep78, or by the way of deleting the intron at the positions of 1907 to 2227 in pCMrep68 and pCMrep40 with the aid of site-directed mutagenesis. The pCMrep78 and pCMrep68 carry two extra mutations which are crucial for the development of the wild-type rep expression plasmid, from which these constructs are derived. The pM1 is proven to be utterly functional with respect to complementation of AAV DNA replication and to inhibition of DNA amplification.

Anti-Rep antibody.

The monoclonal antibody 294-4 is generated by immunizing mice with a bacterially expressed and

gel-purified Rep78 protein that is N-terminally truncated by 171 amino acids. Ascites fluid at a dilution of 1:400 is used for immunoblotting, in which all four Rep proteins are recognized.

Cell culture and virus stocks.

The HeLa and the HeLa-derived cells clones are maintained at 37°C in a concentration of 5% CO2 in Dulbecco's modified Eagle's minimal essential medium supplemented with 5% of fetal calf serum and penicillin-streptomycin. The fetal calf serum is depleted of steroid hormones by activation with charcoal to keep the basal expression level of the MMTV-LTR as low as possible. The adenoviruses of type 2 stocks are prepared. For the preparation of the AAV-2 stocks, we freeze thaw supernatants of AAV-2- and the Ad2-infected cells are cleared by centrifugation and then treated for 30 min at 56°C in the scope to inactivate the helper adenovirus.

Generation of stably transfected cell lines.

Quickly, on the day before transfection, the 2 times 106 HeLa cells are seeded on a dish that has a diameter of 10 centimeters. The pMtrep (20 ug) is linearized with an SspI and is transfected directly. The day after the transfection, the cells are replated at a density of 105 cells per 10-cm f diameter dish. One entire day later, the G418 selection is started at a concentration of 1,200 ug of G418 per ml. The rep-expressing cell clones are subcloned twice by a limitation of dilution.

Transient transfections.

One day before the transfection, 106 cells are plated onto dishes with 10 centimeters of diameter. The cells are transfected by a modified calcium phosphate transfection protocol. After an overnight incubation at 35°C with a concentration of 3% of C02, the cells are washed twice with serum-free medium and then infected with Ad2. After the removal of the virus inoculum, the cells are incubated in a growth medium with 106 M dexamethasone.

Hybridization with labelled oligonucleotides.

To discriminate between the replication of the wild-type AAV and the replication of the rep-negative AAV (pTAV2-3), the two oligonucleotides specific for the region surrounding the BamHI site at position 1045, in a mutated AAV in pTAV2-3, are used: the wild-type oligonucleotide at the positions of 1039 to 1058. 5' GTCCTCCTG GATCCACTGCT 3', which is an antisense, and the pTAV2-3 oligonucleotide at the positions of 1040 to 1059, 5' GCAGTGGATCGATC CAGGAG 3',

which is the sense one. The oligonucleotides are end labelled with 32P and are hybridized at 58°C in 6x SSC, 1 x SSC containing 0.15 M NaCl plus 0.015 M sodium citrate- 5x Denhardt reagent-0.1 mg of yeast tRNA per ml-1% SDS. The filters are washed at 63°C in 6x SSC.

Using the researches in the domain, [1] we decided to create the ITR regions needed for the adenovirus complete replication. They are built by the mRNA construction and ligation (see DNA construction) (Fig. 3).

AAV's Genetic Code

5'—AAUGCgeneticcodeGCAUU—3'

Creation of the AAV

Conform the research conducted by ABM, [1] the virus can easily be created. This methodology gave positive results in virology. This methodology is used here because the virus does not have any fabrication specification. First, we propagate HEK293 cells in PriGrow III Medium (Cat# TM003) with 10% FBS (Cat# TM999) and 1% Pen/strep (Cat# G255). The day before transfection, we plate the cells in a 15cm dish. Then, on the day of transfection, we set up the 3-plasmid co-transfection by diluting 12 µg of helper plasmid, 12 µg of rep/cap plasmid and 10 µg of transgene plasmid in 2.5 ml of serum-free, antibiotic-free medium. Then, the solutions are combined and mixed gently. They are also incubated for 20 minutes at room temperature. Thirdly, after 20 minutes, we add 10.0 ml of serum-free, antibiotic-free medium to the DNAfectinTM2100-DNA complex and we mix the solution gently. After that, we remove the growth medium from the HEK293 cells and add the DNAfectinTM2100-DNA complex solution to the cells. After 5–8 hours, the transfection solution is removed and we add complete growth medium to the cells. The cells are incubated at 37°C in a CO2 incubator for another 48 hours. Around 48 hours after transfection, the cells are harvested from the 15cm plate with a cell scraper. After that, we spin the cells at 1,500 x g for 5 minutes to collect the cell pellet and re-suspend it in 0.5ml lysis buffer (10 mM Tris-HCl (pH8.5), 150 mM NaCl). Then, we freeze and thaw the cell pellet 3 times by rotating through a dry ice/ethanol bath and a 37°C water bath. Finally, the obtained crude lysate is spun down at 3,000 x g for 10 minute and we collect the supernatant fraction

Purification

The research conducted by Addgenes demonstrated that a new way to purify the rAAV viruses was possible. Their methodology is used because the rAAV does not need any special steps in the purification [2].

Cell and Virus Culture.

The replication competent, $E1^+$ Ad, which is the RCA (Rolling circle replication), is detected by the ability to plaque or cause cytopathic effect on monolayers of the A549 cells. The 293-derived cell lines that stably express the Cre recombinase, 293Cre1 and 293Cre4, are propagated in medium supplemented with 0.4 mg/ml G418.

Construction of AdLC8 and AdLC8c Helper Virus.

The plasmids are constructed following the standard protocols. The AdLC8 helper virus is rescued by the cotransfection of 293 cells with pLC8, a plasmid containing a floxed packaging signal, and the pBHG10. The pLC8 is constructed by the following steps:

A 9.6-kb AscI fragment is removed from the pBG18, a plasmid containing the majority of the Ad genome including the Ad packaging signal and the inverted terminal repeats, generating the pLC4. A synthetic loxP site with BamHI compatible terminals, obtained by the annealition of two single-stranded oligonucleotides, is cloned into a pLC4 at a unique BamHI site, located at nucleotide 188 from the left of the Adenovirus 5 genome, generating the pLC5. A second loxP site 9 is inserted into the pLC5 by introducing the loxP oligo into the BamHI site of pABS (Polyclonal antibodies) 9, generating the pLC7 (Phosphoinositide phospholipase C 7) and the subclonation of a 1.4-kb XbaI fragment, containing the loxP site and a neo bacterial-expression cassette, into the unique XbaI site of the pLC5 (Phosphoinositide phospholipase C 5). The resulting plasmid, pLC8 (Phosphoinositide phospholipase C 8), contains thus two loxP sites, separated by 1452 bp and flanked by the Ad packaging signal and neo cassette. The AdLC8cluc helper virus is similar to the AdLC8 one, but doesn't have the neo expression cassette in the E1 (Unimolecular Elimination), and contains the firefly luciferase gene under the regulation of of the human cytomegalovirus (CMV) major immediate-early promoter and a simian virus 40 polyadenylylation signal is inserted in E3 (ubiquitin ligase) with a stuffer sequence.

Construction of the Helper-Dependent Vector,

The pCA35 (principal component analysis 35), which contains the Escherichia coli β-galactosidase (lacZ) ORF (open reading frame) under the control of the murine CMV immediate-early promoter, is digested with SalI, repaired with the Klenow fragment of DNA polymerase, and is recircularized, generating pCA35KS. The murine CMV-lacZ expression cassette is excised from the pCA35KS by digestion with XbaI or BglII and is inserted into XbaI or BamHI. The digestion of the pABS.4 generate the pABS. The 4MClacZ, which is later linearized with SalI and is ligated into the unique XhoI site of pFG140dx3, generating pUMA10R. As a consequence, the pUMA10R retains Adenovirus-specific sequences corresponding to 5789 bp of the left end and 6143 bp of the right end of

the Adenovirus 5. The E1-coding region is disrupted by the insertion of pMX2 at the XbaI site. A 1276-kb SwaI fragment from pUMA10R is removed and replaced by a 8270-bp one from the bacteriophage lambda DNA, generating the pRP1001.

“Rescue” and Amplification of AdRP1001.

The semiconfluent monolayers of the 293 cells in the dishes of 60 millimeters are transfected with 5 µg of pRP1001 for 4 hr at 37°C, and 18 hr after transfection the cells are infected at a multiplicity of infection of 5 plaque-forming units with the AdLC8. Once the medium replaced, the cells are incubated until the monolayers show complete cytopathic effect. The cells are scraped into the medium and the virus is released by three rounds of freezing and thawing. An aliquot of the resulting crude viral lysate of 500 µl is serially passaged on the dishes of 60mm, containing 293Cre4 cells. During each round of amplification of the helper-dependent vector, the 293Cre4 cells are coinfected with the AdLC8 at a multiplicity of infection of 5 for the first two rounds of amplification and at an multiplicity of infection of 1 for subsequent passages. The amplification of the AdRP1001 is monitored by assaying aliquots of crude viral lysate after each passage for lacZ-expressing virus on 293 cells, by counting lacZ-positive cells after 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside staining as follows: 293 cells are infected with the virus inoculum of 500 µl, and are incubated for 24 hr at 37°C. The infected monolayers are washed once with §, fixed with 0.5 ml of 0.2% glutaraldehyde, 2% para-formaldehyde, and 2 mM $MgCl_2$ in PBS^- for 5 min at 37°C, washed, and stained with 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside in PBS^-. The plates are incubated overnight at room temperature, and the blue-forming units are determined. The viral lysates are also monitored for the helper virus and the RCA by plaque assays on 293 and A549 monolayers, respectively. The large-scale virus preparations of the AdRP1001 are prepared by the infection of dishes of 150 millimeters of 293Cre4 cells with 1 ml of crude AdRP1001 stock per dish plus 2×10^7 pfu of helper virus. After the completion of the cytopathic effect, the purification of AdRP1001 virions is performed by the CsCl (cesium chloride) buoyant density centrifugation, and the fractions are collected through the viral bands, and are assayed for luciferase-expressing virus after a dilution of 1:1000 and the infection of 293 cells.

Injection Solution

- Conservation agents;
- Formaldehyde
- Phenol
- 2-phenoxyethanol
- Thimerosal
- Antibiotics: neomycin et polymyxin B

1. Stabilators;
2. Bovine albumin or bovine serum
3. Human albumin
4. Gelatin
5. wistaria
6. Lactose
7. sorbitol
8. Sucrose or sucrose
9. Polysorbates 20 or 80

Results

In Vitro Synthesis of Modified mRNA for Induction of Protein Expression in Human Cells

Construction of the DNA

For the creation of the DNA, it is necessary to include the plasmid DNA into an E. coli culture and to expose it to several chemical and thermal reactions. After that, we obtain a complete DNA containing the CAP and Rep genes and the ITR regions.

Transcription of the DNA

To be transcript, the DNA has to be exposed to different enzymes and to be exposed to a variation of the temperature. We obtain a full mRNA code containing all the information described in the previous section.

Manufacturing of Cap genes

Construction of VP1/VP3

After the expression of VP1 and VP3 in E coli, chemicals reactions in the culture of the E coli provoke the apparition of VP1/VP3. Then, the mixture is assembled with mRNA. We obtain a VP1/3 genomic complex without the ACKR3 genetic code.

Assembly of VP1/VP3 with the mRNA

To be assembled, these elements must be incubated and to be exposed to different organic elements. We obtain a fully operational complex of VP1/3 DNA.

Assembly of VP2

For the assembly of the VP2 complex, it is necessary to use fetus cells. The Genomic DNA is included in cells to be replicated and to impose the cells to create the VP2 complex. Bacmid is created to be used for the creation of baculoviruses containing the VP2 sequences. After that, the VP2 complex was extracted by a virus. Then, the virus containing VP2 was eliminated and the VP2 was used. We obtain a VP2 genomic code.

Manufacturing of Rep genes

The construction of the pP5-repcap-Neo plasmid used to generate the A549-based rep and cap cell lines and the stable transfection, which is the G418 selection, and the screening of initial clones are described. We further need to subclone the initial positive clone, which is called N43, by a series of dilutions, the G418 reselection, and the clonal expansion in 96-well plates, 24-well plates and plates of 100 millimeter. We rescreen individual subclones for production of the AAV by the transfection method. We also obtain a REP genetic code which will code the virus replication in the cell.

Plasmid constructs and transfections

We generat the pAAVCMVGFP construct by replacing the Xbal fragment of pSub201 plasmid with a DNA fragment containing the cytomegalovirus early promoter, the enhanced green fluorescent protein gene, and a simian virus-40, having a poly A signal. We carry out all transient transfection experiments by the usage of Effectene transfection reagent, in accordance to the manufacturer's instructions.

Evaluation of AAV production in rep/cap cell lines

We examine the production of the AAV in rep and cap cell lines by either the staggered infections of helper adenovirus and the Ad-AAV hybrid vector. For the transfection process, we pre-infect the cells with the adenovirus at appropriate MOIs one day before the transfection with the vector plasmid. The production of the AAVCMVGFP per cell is determined on 84-31 cells.

DNA hybridization

We prepare a total cellular DNA using the Qiagen Genomic-tip, which is 20/G, in strict accordance to the manufacturer's instructions. We analyze the DNA samples of 10 µg for rep and cap sequences following digestion with either BamHI. We hybridize the blots to 32P-labeled probes, either a 2.7-kb cap or an 840-bp rep probe.

Creation of the rAAV

To create the AAV, it was necessary to add into cells HEK293 the AAV's genome (REP/CAP genes) and a helper virus. The helper virus was AdLC8.

Purification

The virus AdLC8 was rescued by the protein pLC8, plasmid containing a "floxed" packaging signal, and pBHG10. AdRP1001 was amplified and rescued by several chemical reactions and by the centrifuge force.

Discussion

The VP1/VP3 complex is responsible of the entrance of the virus in a cell. They contain the amino-acids code of the mutated ACKR3 contact protein. They are important because they are the only way to force the virus to enter in cancerous cells only.

Based on the research of "The American Society of Gene Therapy", I found that VP2 is not essential in the cure. Yet a VP2 complex can easily be created and used without any consequences.

DNA is a major part of the cure. The DNA is used to create the VP1/VP3 capsid protein (see above). When the DNA is synthetized, it has to be transcript into a mRNA code for the protein expression.

When it is performed, it is possible to assemble the mRNA code with the VP1/VP3 complexes to obtain the adapted VP1 and VP3 complexes (contain the ACKR3 amino-acids code).

The Rep genes are responsible of the replication of the modified Adenovirus. That implies the REP genes do not have to be specifically adapted. It is why the standard protocol is used in the research.

The rAAV is designed to deliver its DNA to the cancer cells. By delivering its DNA, it will force the cancerous cells to create other Adenovirus and finally it will kill theme by lake of ATP.

The purification is necessary and extremely important because the helper gene can be dangerous for the patient's health.

Also, the efficiency of the adenoviruses to deliver a transgene is limited by the use of classical biological methods. However, this new hypothetical treatment does not require a transgene, this is why it is not affected by the major limitation.

Finally, the immune response is very small [1]. According to ABM, the low damages that the Adenovirus can potentially cause and its low pathogenicity leads to a very mild immune response. This propriety made it the best candidate for a potential cancer cure.

Conclusion

In the near future, due to numerous environmental and genetical factors, the number of new cancer cases will significantly increase, demanding a new way to treat this disease.

As the research has demonstrated, it is possible to treat the cancer with an Adenovirus made in laboratory. This virus contains the REP and CAP genes closed by the ITR regions. This treatment permits an effective cure of all types of cancers. It will, however, take time and money. Order of the needed equipment, synthesis of the REP and CAP genes are all the steps which are necessary to develop a rAAV which would treat the cancer.

The usage of the Adenovirus will greatly increase the success percentage of healing from a cancer affecting the designated organs *(see introduction)*. Also, their will be no need to use the radiotherapy, the chemotherapy, the surgery, the immunotherapy, the hormone therapy or the stem cells transplant, which does not provide a success of 100 percent and may have secondary effects that can negatively affect the lives of the patients.

Abbreviations

1. SOC: Super Optimal Broth
2. LB: Luria broth
3. µg/ml: micrograms per milliliter
4. 37 °C.: 37° Celsius degrees
5. *coli: Escherichia Coli*
6. (10 pg to 100 ng)
7. Min: minutes
8. Sec: seconds
9. Rpm: rotations per minute
10. 1 hr : 1 hour
11. CDS: coding DNA sequences
12. poly T-tail: Poly thymine tail
13. PCR: Polymerase Chain Reaction
14. IVT: in vitro transcription
15. eGFP: Green fluorescent protein
16. T: thymidines
17. NTP/cap: Nucleosidtriphosphate/cap
18. DPBS: Dulbecco's phosphate-buffered saline
19. IPNV: infectious pancreatic necrosis virus
20. EDTA-free: Ethylenediaminetetraacetic acid-free
21. Tris: trisaminomethane
22. IPNV RdRP : infectious pancreatic necrosis *RNA-dependent RNA polymerase*
23. ATP: Adenosine triphosphate
24. GTP: Guanosine-5'-triphosphate
25. CTP: Cytidine triphosphate
26. UTP: Uridine-5'-triphosphate
27. Pmol : picomole

28. PPV: porcine parvovirus
29. PPV-VP2 FD: porcine parvovirus *VP2* protein
30. sf9: Spodoptera frugiperda 9
31. IMAC: Immobilized metal affinity chromatography
32. anti-PPV: anti porcine parvovirus
33. MMTV-LTR: Mouse mammary tumor virus long terminal repeat
34. P-globin: platypus β-*globin*
35. SDS: Sodium Dodecyl Sulfate
36. RCA: Rolling circle replication
37. pABS: Polyclonal antibodies
38. pLC7: Phosphoinositide phospholipase C 7
39. pLC5: Phosphoinositide phospholipase C 5
40. pLC8: Phosphoinositide phospholipase C 8
41. E1: Unimolecular Elimination
42. CMV: Human cytomegalovirus
43. E3: ubiquitin ligase
44. pCA35: principal component analysis 35
45. ORF: open reading frame
46. CsCl : cesium chloride

Declarations

Acknowledgements

I would like to express my very great appreciation to Dr Ben Berkhout for his valuable and constructive suggestions during the planning and development of this research work. His willingness to give his time so generously has been very much appreciated.

Declaration of Interests

This article does not contain any studies with human participants performed by any of the authors.

I declare that I have no conflict of interest and that I consent for the publication of this manuscript.

I also acknowledge that I shall make another declaration to state any change in any matter contained in this declaration within one month after the change occurs and shall provide further information on the particulars contained in this declaration if so required by the Treatment of 27 kind of cancer research program.

I hereby declare that I have no pecuniary or other personal interest, direct or indirect, in any matter that raises or may raise a conflict with my duties as a manager of the Treatment of 27 kind of cancer research program.

This study was not funded.

I made a substantial contribution to the conception, design of the work; the acquisition, analysis, interpretation of data; the creation of new software used in the work; have drafted the work and substantively revised it.

I would like to express my very great appreciation to Dr Elena Tap for his valuable and constructive suggestions during the planning and development of this research work. His willingness to give his time so generously has been very much appreciated.

I would also like to thank the staff of the following organization for enabling me to visit their offices to observe their daily operations:

Ion Creangă Pedagogical State University

References

1. ABM: https://www.abmgood.com/marketing/knowledge_base/Adeno_Associated_Virus_Production_and_Modification_of_AAV.php
2. Addgenes: https://www.addgene.org/protocols/aav-purification-iodixanol-gradient-ultracentrifugation/
3. ExPASy:https://web.expasy.org/translate/
4. Nature: https://www.nature.com/articles/2241083a0
5. NCBI: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4354090/

6. NCBI: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3592137/
7. New England Biolabs
8. PasseportSanté: https://www.passeportsante.net/fr/Actualites/Dossiers/ArticleComplementaire.aspx?doc=vaccination-que-contiennent-les-vaccins_do
9. Translate Tool: Lhttps://skaminsky115.github.io/nac/DNA-mRNA-Protein_ Converter.html
10. UniProt: https://www.uniprot.org/uniprot/P25106

Figures

Figure 1

Needed DNA fragment for VP1 genes

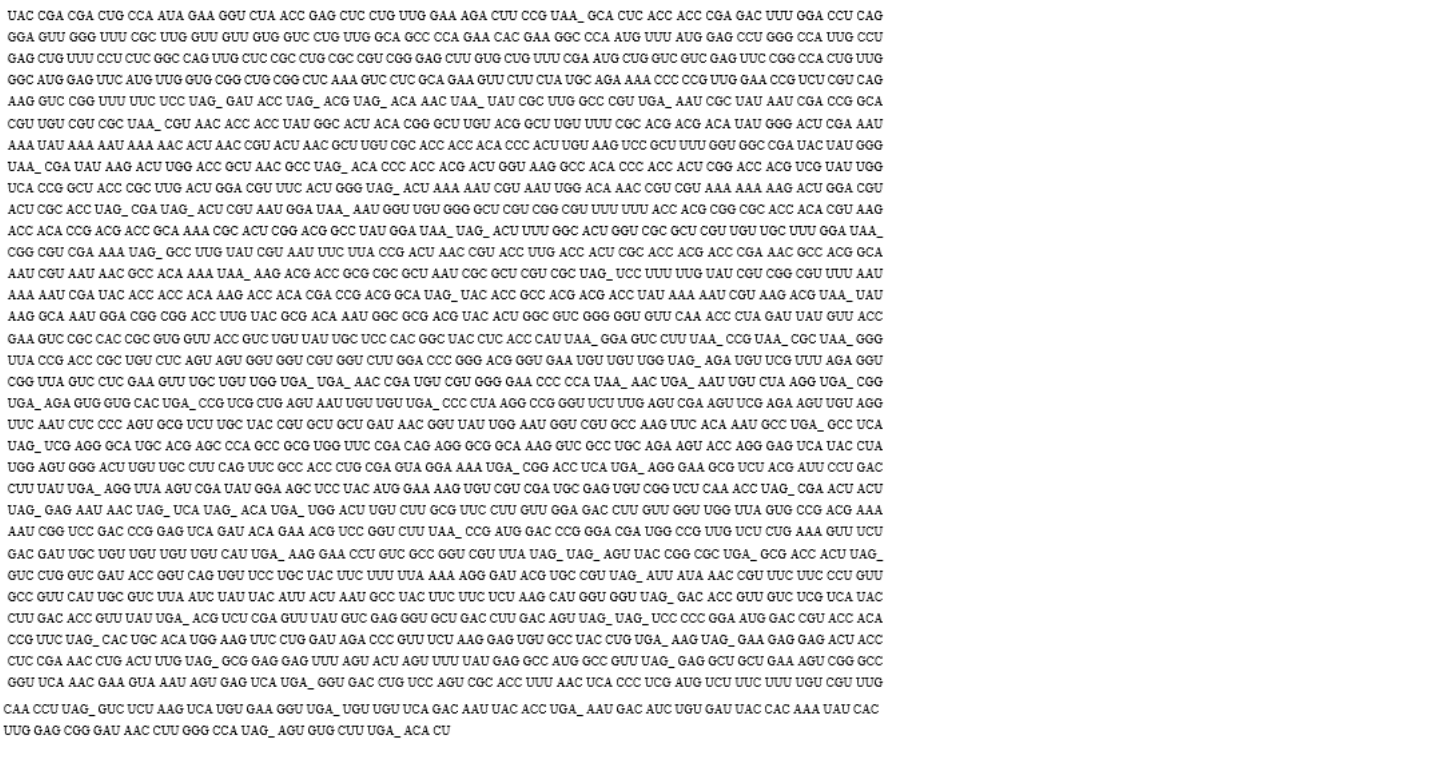

Figure 2

Needed VP1 genetic code

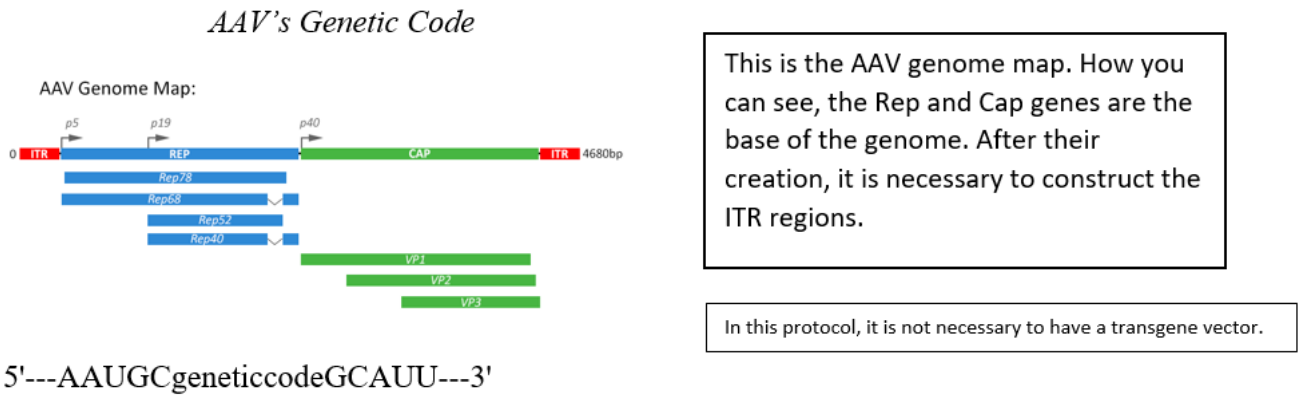

Figure 3

AAV's Genetic Code