Recombinant DNA and genetic engineering

Genetic engineering

- a set of techniques used to c**hange the gene composition or to modify the genome organization of cells/organisms**
- includes transfer of genes within and across species boundaries to produce organisms with improved properties or novel functions (utilization in research, medicine, agriculture and biotechnology)
- transport of genes is mediated by recombinant DNA; whose synthesis is usually the first step in all procedures

Recombinant DNA

- an artificially (in vitro) synthesized DNA, usually composed of two basic parts (originated from different organisms): the gene of our interest inserted in the vector DNA
- vectors are the most frequently derived from bacterial plasmid or virus (bacteriophage, retrovirus) and allow the transfer of gene to the new organism
- the process of synthesis and multiplication of recombinant DNA used to be called cloning of DNA

Plasmids

- extrachromosomal DNA of bacteria
- small circular molecules of DNA (1-250 kbp)
- usually one large plasmid or more small plasmids per bacterial cell
- independent – replicate separately from the main chromosome

Cloning of DNA

- allows to isolate the particular gene or sequence from the genom, to multiply it in the form of recombinant DNA and to make it thus accessible for further applications (isolation and studies of genes and regulatory sequences; expression and overexpression of foreign genes/recombinant proteins; production of genetically modified or transgenic organisms, in which the cloned foreign or modified genes can be permanently incorporated in genomes; and many others)
- production of genomic libraries is also possible due to cloning of recombinant DNA; genomic library is a collection of cloned fragments (inserted in one type of vector), which are prepared by cutting of genomic DNA and all together represent the total genom of a single organism; genomic library is usually kept in the form of bacterial clones, where each bacterial clone carries different fragment of genomic DNA

(the individual genes can be found within the genomic library via method of hybridisation using specific probes)

- cloning of DNA includes three main steps:
  1. preparation of recombinant DNA
  2. transfer of recombinant DNA into the host cell
  3. selection of clones containing recombinant DNA

1. **Preparation of recombinant DNA:**
   - **isolation of appropriate gene:**
   1. using genom DNA from the donor organism: the gene of our interest is separated from the rest of the genom via cutting by restriction endonucleases or (more often) amplified by Polymerase chain reaction (PCR)
   2. using cDNA (complementary DNA): cDNA is synthesized as complementary strand from mRNA of donor organism by reverse transcription; used especially for heterologous expression of eukaryotic genes in prokaryotes (bacteria don't use introns and splicing modifications, the expression of eukaryotic genomic genes in prokaryotes is thus not always functional; using cDNA without introns usually solves this problem)
   - **vectors (cloning vectors):**
     - as they are mostly derived from bacterial plasmids and viruses, they have the ability of autonomous replication within the host cell and allow thus the transfer and cloning (multiplication) of inserted gene
     - vector prepared from plasmid must have two extra characteristics:
       1. multiple cloning site (polylinker): a short segment of DNA containing many (up to 20) restriction sites (= the sites for restriction endonucleases), which are typically unique
       2. marker genes, whose products allow (on the base of its phenotypic manifestation) the selection of right clones (= clones carrying the vector with inserted gene); typical marker genes are the genes determining the antibiotic resistance
     - expression vectors: cloning vectors having, in addition, strong promoter localized upstream of the polylinker; the product of inserted gene is thus expressed in high levels; used in biotechnology for production of recombinant proteins (high concentrations of product can be toxic for the host cell, the promoters used are therefore often inducible = switched on after the culture has grown to the optimal density)

- except of plasmid vectors, the vectors derived from bacteriophages (lambda, M13) are often used for molecular cloning
  - **yeast artificial chromosomes (YAC):** are able to replicate in both bacterium and yeast and have high cloning capacity
  - finally, both the gene and the vector are cut by the same restriction endonuclease (with the unique site at polylinker) and link together covalently by ligase - the chosen gene is thus inserted in the vector at the place of polylinker - the recombinant DNA is prepared for transfer to the recipient organism
  - **restriction endonucleases:**
    - enzymes that cleave dsDNA at the place of (or near) specific and usually symmetric (palindromic) sequence (recognition or restriction site)
    - natural enzymes of bacteria, the component of restriction modification system, which provides a defence against foreign (e.g. bacteriophage) DNA (the majority of viral DNA is cut; the bacterial own DNA is protected by methylation)
    - the name of each enzyme is derived from the bacterium, in which it was discovered (e.g. EcoRI from *E. coli*)
    - each restriction endonuclease has its specific recognition site (usually 4-6 nt) in which it cut forming either cohesive or blunt ends

2. Transfer of recombinant DNA to the host cell:
   - host cells: the most significant are bacteria (at the first place E. coli) and yeasts; in some cases also insect or mammalians cells can be used (in general, suitable host cells are the cells which are characterised by fast reproduction and in which the recombinant DNA (= the inserted gene) can be cloned and multiplied significantly in a short time)
   - methods of transfer: in case of bacterial vectors, the methods are based on the induction of competence state followed by transformation of bacteria – the recombinant DNA enters the host cells (chemical ways followed by heat shock; electroporation and some other techniques); viral vectors enter the recipients via process of infection; for animal cells, the microinjection is also used
   - to confirm the presence of the new gene in recipient organism and to determine the level of its expression, alternatively to test its chromosomal location and copy number in case of inserted gene, different methods can be used: PCR, Southern hybridization, DNA sequencing, northern hybridization, quantitative RT-PCR, Western blot, ELISA and some others

Applications of genetic engineering

- in biotechnology: industrial production of recombinant proteins (e.g. coagulation factors for therapy of haemophilia), vaccines (against hepatitis A and B, against influenza etc.), human hormones (insulin, somatotropin, somatostatin, etc.), amino acids, enzymes, alkaloids, steroids etc. using cloning and protein expression systems; transformed organisms (usually bacteria and yeasts) are cultivated in bioreactors (microbial fermentation); the overexpressed protein is then purified
- **in medicine:**
  - experimental gene therapy (some cases of gene therapy also in clinical research):
    - particularly for cancer and monogenic hereditary diseases
    - generally replacing defective genes with effective ones (but the genetic manipulations can also aim, especially in case of cancer, to the targeted inhibition of gene expression or targeted destruction of affected cells)
    - use of recombinant viral (retroviral, adenoviral) vectors, which have most of their viral genes replaced by the human gene of interest; viral vectors are able to introduce the gene of interest into the affected cells (in case of retroviruses, the gene is integrated directly into the recipient's genome)
    - problems: different level of efficiency; random insertion into the genom can switch on the process of malignant transformation; temporary and low expression; immune reaction of recipient against virus (in case of repeated application)
  - by genetic modifications of pathogen (bacterium or virus), the live attenuated vaccine can be produced
  - genetic modifications of antigens and antibodies are made to strengthen the immune response
  - genetic engineering is used to create animal models of human diseases (cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging, Parkinson disease), against which the potential cures can be tested; the most common model are genetically modified mice (genetically modified pigs are tested for pig to human organ transplantations)
- **in research:**
  - revelation of sequences of many eukaryotic genes (sequencing projects)
  - study of gene expression and function
  - creation of new models of human diseases in animals:
  - transgenic organism: an organism having the gene from other species (= transgene) inserted in genome; the gene has been transferred into the germ line cells, therefore all cells of transgenic organism possess the inserted heterologous gene
  - transgene can be integrated to the genome of recipient organism and become its permanent part (e.g. using the retroviral vector for transfer) or stays extrachromosomal (not inserted in genom) and its presence and expression is usually temporary (e.g. using if adenoviral vectors)
  - **possible applications:**
    1. insertional mutagenesis: transgene integrates randomly to some gene of recipient and causes thus its inactivation
    2. replacement of the defect gene with the functional copy (via homologous recombination inside of recipient)
    3. replacement of the functional gene with non-functional copy (homologous recombination) = the

production of knockout organism (= organism, which is engineered to lack the activity of one or more genes); knockout organisms are used as models of different human diseases or allow to reveal the function of not completely known genes

4. not only genes coding for protein product, but also regulatory sequences can be transferred – for example the sequence of antisense RNA (microRNA), which can inactivate specific mRNA in recipient organism on the base of complementarity