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Construction of VEGF encoding mini-circle plasmid and development of a semiquantitative RT-PCR measuring VEGF-mRNA

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Title (English)

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Abstract

Bacterial sequences in plasmid-based vectors for gene therapy are believed to inhibit long-term gene expression. In this master thesis, a novel recombination method was used to produce a mini-circle plasmid consisting of the expression cassette for hVEGF. The expression of hVEGF from this plasmid, devoid of bacterial sequences, was compared to that of an existing hVEGF-plasmid using ELISA. The mini-circle proved to have an equal or possibly better expression of hVEGF. A method for measuring VEGF-mRNA levels in cotransfected cells with a semiquantitative RT-PCR was developed.

Keywords

Gene therapy, plasmid based vectors, mini-circles, hVEGF, semiquantitative RT-PCR

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Construction of VEGF-encoding mini-circle and development of a semiquantitative RT-PCR measuring VEGF-mRNA

Sofia Stenler

Sammanfattning

I genterapi finns två system för att föra in gener i målcellen: System baserade på virus, och system baserade på DNA-cirklar framställda i bakterier, så kallade plasmider. För att plasmiderna ska kunna växa i bakterier måste de innehålla bakteriella gensekvenser samt en selektionsmarkör. Selektionsmarkören är en DNA-sekvens som gör att bakterien måste behålla plasmiden, ofta en antibiotikaresistensgen. Undersökningar har emellertid visat att de bakteriella sekvenserna gör att genen tystas när den nått målcellen. Dessutom önskar man minska spridningen av antibiotikaresistensgener. Därför vore det önskvärt med en plasmid som inte innehåller några bakteriella sekvenser och ingen antibiotikaresistensgen, utan bara det som krävs för att genen i fråga ska uttryckas i målcellen. I mitt examensarbete har jag framställt just en sådan plasmid, genom ett snillrikt system från Stanford, USA. En stor plasmid, i bakterien, delar sig i två mindre: En minicirkel utan bakteriellt DNA och en större med de icke önskvärda sekvenserna. Genuttrycket i cellkultur från denna minicirkel har jämförts med genuttrycket från en existerande större plasmid. Man kan inte säga att det säkert finns en signifikant skillnad i genuttrycket mellan de båda plasmiderna, men minicirkeln är åtminstone lika bra som den större plasmiden. Jag har även utvecklat en metod för att mäta genuttrycket på mRNA-nivå.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet, april 2006

Abbreviations

Amp – ampicillin
cDNA – complimentary deoxyribonucleic acid
CMV – cytomegalovirus
DMEM – Dulbecco's modified Eagle's medium
EGFP – enhanced green fluorescent protein
ELISA – enzyme-linked immunosorbent assay
HSV – herpes simplex virus
hVEGF – human vascular endothelial growth factor
IF1 – translation initiation factor 1
IHD – ischemic heart disease
Km – kanamycin
LB – Luria-Bertani broth
mc – mini-circle
ORT – operator-repressor titration
PEI – polyethylenimine
PNA – peptide nucleic acid
Q-RT PCR – quantitative reverse transcriptase polymerase chain reaction
RT-PCR – reverse transcriptase polymerase chain reaction
ssDNA – single stranded DNA
TB – terrific broth
VEGFR – vascular endothelial growth factor receptor

Introduction

1.1 Aim

The aim of this master thesis was to produce and test a small plasmid based vector for gene therapy. The plasmid is a mini-circle containing only the expression cassette for human vascular endothelial growth factor (hVEGF). This is desirable in order to lessen the spread of antibiotic resistance genes and the introduction of bacterial DNA into eukaryotic cells. The bacterial sequences are thought to repress the long-term expression of the gene.

The mini-circle was formed through recombination of a larger plasmid carrying the the gene for a recombinase as well as expression cassette between the two recombination sequences. The expression of hVEGF from purified mini-circle was measured and compared to that of an existent larger plasmid carrying the same expression cassette. Expression was measured in cell lines and quantified with ELISA. A method to measure the mRNA-levels through a semi-quantitative PCR was developed.

1.2 Gene therapy and vectors

To be able to mend a human at her very core, her genome, is an alluring thought. In theory, gene-therapy is beautifully simple. Replace a broken gene with a healthy one, or enhance the function of a cell with a brand new one. In practice, it is not quite so easily achieved. One of the major obstacles is the delivery of the gene into the cell-nucleus. There are two principal ways of transferring the desired gene into the cell, two types of vectors: viral and non-viral.

1.2.1 Viral vectors

Viruses have fine-tuned the ability to transfer nucleic acid into the nucleus of a cell for millions of years. Hence, the viral vectors are a straightforward way for gene therapy. The most commonly used viruses are recombinant retrovirus, adenovirus and herpes simplex virus (HSV). The retrovirus integrates DNA into the host-genome. The retroviral vectors for gene therapy also show this feature, the delivered gene will be constantly present in the cell and all daughter cells. This causes a persistent expression of the gene. Therefore, retroviral vectors are useful for long-term treatment. Adenoviruses and HSV do not integrate their genome into the host genome; hence, the expression will not be stable. This can be desirable in the case of short-term treatments. The transfer rate is high for most viral vectors.[1]

There are however some problems with viral vectors for gene therapy. Apart from the fact that the viral vectors could somehow regain replication competence or even virulence, high titers of retroviruses is hard to produce. Even though the persistent gene-expression of retroviral vectors is desirable, there have been cases of insertional mutagenesis.[2] It is not possible to control where in the genome the gene is inserted. Children suffering from severe combined immunodeficiency (X-linked SCID) were quite successfully treated with the gene for γc interleukin receptor subunit in a retroviral vector. Unfortunately, three out of fifteen developed cancer; the vector integrated in a proto-oncogene. The gene was then expressed and the children developed cancer. The adenoviral vector is not integrated into the genome; hence, there is no risk of insertional mutagenesis. However, repeated treatments might be necessary if expression over a longer period is desired. This is a risk factor for immunoreactions, especially

[1] Emery (2004) p. 413
[2] Ibid. p. 418

as adenovirus is a common human pathogen; many patients already carry antibodies. An immunoresponse might not only harm the patient, but it may also prevent a successful gene transfer.[3]

1.2.2. Plasmid based non-viral vectors

Non-viral vectors are produced in bacteria as plasmids, normally in *Escherichia coli.* The vector consists of the gene expression cassette and sequences needed for propagation in prokaryotes. It can be delivered as a naked piece of DNA or together with different types of transfection reagents to facilitate the uptake. The gene transfer rate is not as high as with viral vectors though. [4]The plasmid has to pass the cell membrane, cytoplasm and the nucleic membrane into the nucleus. The gene will not be integrated into the genome but exist as an episome. Hence, the gene expression will diminish in time as the cells divide.

To be able to produce plasmids in bacteria one must use a selection marker. This is commonly a gene for antibiotics residence, e.g. ampicillin (Amp). However, large amounts of antibiotics in production can be costly. There have also been cases of allergic reactions to antibiotics as traces of antibiotics have been found in the purified product. One must also consider the risk of further spread of resistant bacteria. It would be desirable to have a plasmid vector that does not require the use of antibiotics, or at least does not contain the gene for resistance in its final form. Another problem with plasmid-based vectors for gene therapy is the low and transient expression of the gene delivered. Chen *et al.*[5] have studied the effect of the bacterial sequences on the gene expression of non-viral vectors. They have shown that gene expression is silenced if the expression cassette is covalently linked to bacterial sequences. The cell might recognise certain elements in the bacterial DNA such as CpG-sequences, which are believed to be immunogenic.[6] A plasmid without bacterial sequences would be desirable.

Consequently, the aim of the game is to produce small circles of the expression cassette devoid of bacterial sequences and the gene for resistance to antibiotics, which then will have a more long-lived expression. Naturally, the expression must be compared with some standard plasmid and the hypothesized difference in expression has to be verified.

1.3 Cardiovascular diseases and vascular endothelial growth factor

Cardiovascular disorders are an all too common cause of distress, disease and death. The VEGF is a family of proteins interacting with three receptors (VEGFR). They all take part not only in various diseases where there is a disorder of the vasculature, e.g. ischemic heart disease (IHD), peripheral artery disease, but also in tumour angiogenesis in cancer.[7]

VEGF binds VEGFR-1 and -2 as well as two neuropilins, a type I transmembrane protein[8] which acts as a transmembrane co-receptors to VEGF-A[9]. The growth factor induces proliferation, sprouting, migration and tube formation of endothelial cells. It also enhances permeability of the endothelium and plays a part in the increase of nitric oxide production. Furthermore, VEGF stimulates the inflammatory cell recruitment and interacts with

[3] Emery (2004) p. 414
[4] Ibid p. 413
[5] Chen (2004)
[6] Liu (2004)
[7] Tammela (2005) p. 556-558
[8] Kolodkin (1997)
[9] Fukasawa (2004) p. 3327

hematopoietic stem cells, among others. VEGF-expression is induced by several cytokines. [10] There exists at least six different splice-isoforms of VEGF. In this study, I have worked with the $VEGF_{165}$, which is one of the major secreted forms[11]. After secretion, it binds to the cell surface heparin sulphate proteoglycans.

VEGF might be used to promote the collateral vessel formation in IHD. There have been studies where IHD was treated with adenoviral vectors for VEGF as well as with naked plasmids. They both gave a transient expression, which is favourable since one does not want an angiogenesis growth factor to be permanently over-expressed. It could awaken tumour cells. Studies imply that VEGF-treatment increases nutritive delivery of arterial blood to ischemic muscular tissue of the heart.[12]

1.4 Overview of the methods used

1.4.1 Cloning

When cloning plasmid vectors, one utilizes restriction endonucleases and ligation enzymes to form clones and grow them in bacteria. Restriction endonucleases cleave DNA at a specific sequence, making it a useful tool for bioengineers. There are enzymes that cleave both strands at the same position, leaving a blunt end, and those that cleave one strand a few bases away from the other, leaving a so-called sticky end. The sticky ends will ligate only with complimentary sticky ends, where as the blunt ends can be ligated to any other blunt end. A sticky end can be made blunt by the use of a DNA polymerase I Klenow-fragment, which fills in the missing bases or cuts off the unpaired bases.

When constructing new clones, a fragment is inserted into an existing vector to change the sequence. The vector and the insert are cleaved with matching restriction endonucleases. It is common to dephosphorylate the ends of the vector to prevent self-ligation. In this way, ligation preferentially occurs between the vector and the insert, which still has a phosphate for the ligation enzyme to use. This will reduce the vector background in cloning assays. The ligase used is usually T4 DNA ligase, which needs ATP to catalyse the formation of a phosphodiester bond between the 5' phosphate and 3' hydroxyl termini in duplex DNA or RNA.

1.4.2 Human cell lines

Eukaryotic cells are more difficult to grow in culture than bacteria. Growth of these cells requires a complex media containing serum and some amino acids. There is a high risk of infection by fungi or yeast. In order to obtain cells that can grow and divide unlimited number of times, the cells must be immortalized. Their growth properties are then transformed via a virus, e.g. papilloma or adenovirus. Some cell lines are adherent, i.e. they need a surface to grow upon and must not be grown to a too high cell density.

In this thesis, we used human cell lines as we wished to study the expression of hVEGF. HeLa cells origin from a woman named Henrietta Lacks, who suffered from cervical cancer. She died in 1951 and ever since, these cells have lived on in various laboratories all over the world. HeLa cells are adherent cells that are quite easy to grow *in vitro*. HT-1080 is another adherent human cell line. It originates from a fibrosarcoma tumour in connective tissue.

[10] Tammela (2005) p. 551-552
[11] Ibid p. 551
[12] Blomberg (2003) p. 946

1.4.3 Enzyme-Linked Immunosorbent Assay (ELISA)

To detect proteins produced by cells in culture it is common to use the ELISA method. In principle, an enzyme-labelled monoclonal antibody, specific for the protein in question, attaches to its epitope on the protein and produces a signal that can be quantified. The signal is the activity of the enzyme coupled to the antibody. After washing off unbound antibodies, a substrate for the enzyme is added and the amount of coloured product is measured after a certain time. The intensity of the colour will be proportional to the amount of bound protein. An even more specific method is the sandwich ELISA where a monoclonal antibody is used to capture the protein and a second, polyclonal antibody is labelled with the enzyme.

1.4.4 Polymerase chain reaction (PCR)

A polymerase chain reaction is a method to amplify a relatively short fragment of double stranded DNA. The DNA is melted and two short oligo-nucleotides, specific for sequences flanking the region to be amplified, bind the ssDNA. The thermostable polymerase uses these primers to attach to the DNA and start building the complementary strand. These three steps, melting, annealing and extension, are repeated several times, e.g. 35. For each repetition, there is an exponential increase of copies of the sequence in question. Hence, PCR is a very sensitive method for detecting a specific DNA sequence. Since there will be no exponential amplification of the product unless both primers anneal, it is also specific. Its specificity is limited by the specificity of the primers.

1.4.4.1 Reverse transcriptase PCR (RT-PCR)

To be able to amplify RNA, one must first convert it into cDNA. This is done before the actual PCR, either in a separate reaction or in connection to the PCR. The latter is to be preferred, because the less the samples are handled, the better. In this “first strand reaction”, one of the primers is used as the start point for a reverse transcriptase. Once enough cDNA is believed to have been generated, the reverse transcriptase is heat-inactivated as the polymerase is activated. The cDNA then acts as template for the standard PCR. RT-PCR is one of the most specific methods for detecting mRNA.

1.4.4.2 Semi-quantitative PCR

A common method to analyse PCR product is to run the samples on an agarose gel containing ethidium bromide. The desired product is seen as a band of the expected size. This however is not a quantitative method for detection. Once the PCR-reaction has gone to an end, the amount of product will reflect the initial concentration of dNTP and primers rather than that of the template. In a semi-quantitative PCR, the reaction is stopped while still in the exponential phase. The exponential phase of amplification occurs in the PCR cycles when reaction components are still in excess and the PCR products are accumulating at a constant rate. Hence, the signal from the sample run on an agarose gel and stained with ethidium bromide will reflect the initial amount of template, and can be quantified and used in estimations of mRNA levels.

1.4.4.3 Quantitative PCR (Q-PCR)

In order to more specifically quantify the amount of template, data must be collected at several points during the PCR. This is done in real time in a Q-PCR. Here one measures a substance that signals if it is bound to DNA or not. This is often a DNA-probe designed to bind to the sequence somewhere between the primers. It will bind the DNA after the double strands are melted and be cleaved or pushed away by the polymerase building the other strand of the fragment. Hence, the signal from the probe will be proportional to the amount of PCR product

in the sample. The signal is measured throughout the PCR reaction. After constructing a standard curve from known concentrations, the initial concentration of the template can be determined. It is a very sensitive method, and the annealing of the probe adds to the specificity.

2. Materials and methods

2.1 Plasmids and cloning

2.1.1 phVEGF165.SR

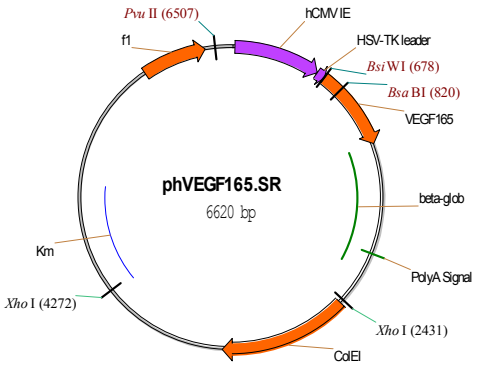

Figure 1. Plasmid phVEGF165.SR

The plasmid phVEGF165.SR contains an expression cassette for hVEGF. The expression is driven by a human cytomegalovirus (CMV) promoter, and is followed by a polyadenylation sequence from the rabbit β-globin gene. As can be seen in fig. 1, the plasmid also contains a translation initiation signal, namely an un-translated herpes simplex virus (HSV) thymidine kinase sequence upstream of the VEGF-gene. The selection marker chosen for this plasmid is a kanamycin (Km) resistance gene. The plasmid size is 6620 bp. [13] It was constructed by Genentech, CA, USA.

2.1.2 p2ΦC31

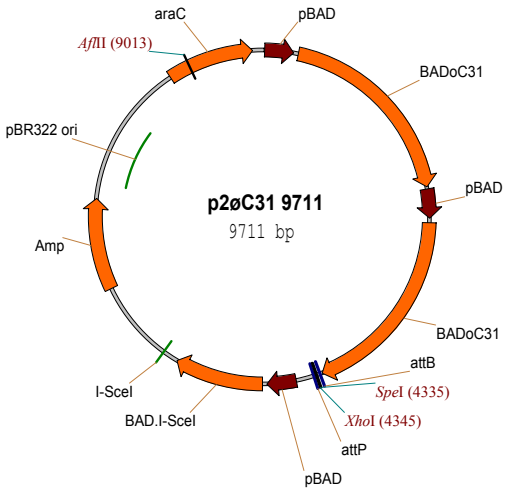

Figure 2. Plasmid p2ΦC31

[13] Isner (1996)

The p2ΦC31-plasmid seen in fig. 2 has a system for a *Streptomyces* temperate phage integrase ΦC31-mediated recombination.[14] It contains two copies of the gene encoding the ΦC31 integrase, and its specific recombination sequences, *att*P and *att*B. The plasmid also contains the gene for a restriction endonuclease with a very rare recognition site, *I-Sec*I, as well as the sequence for the restriction site. All genes are controlled by the BAD-promoter. This promoter is activated through the addition of L-(+)-arabinose. The selection marker for this plasmid is the Amp resistance gene. The plasmid size is 9711 bp. The plasmid was kindly provided by Mark Kay, Stanford, CA, USA.

2.1.2 Cloning

Escherichia coli DH5α was used for the cloning of VEGF-fragment from phVEGF165.SR into p2ΦC31. The insert was cleaved by *Pvu*II and *Xho*I. The plasmid was cleaved by *Xho*I and *Spe*I. The latter end was made blunt with Klenow. Inserts and vector were gel-extracted and purified using a Jet quick gel extraction kit manufactured by Genomed, MO, USA. The insert was ligated into the vector by T4 DNA Ligase. This plasmid is annotated p2ΦC31_hVEGF, and the mini-circle resulting from recombination is named mc_hVEGF. The plasmid was transformed by heat-chock into *E. coli* DH5α according to a protocol from Invitrogen, CA, USA.

To be able to distinguish between mRNA from mc_hVEGF and phVEGF165.SR, two mutated versions were needed. The protein should however not be affected, so the mutations had to be silent. Each silent mutation introduces a cleavage site for a new restriction enzyme. One mutant bears a site for *Bfa*I, the other for *Hae*III. For the DNA sequences, see fig. 3. *E. coli* DH5α was used for the cloning of the mutated fragments into phVEGF165.SR. The designed fragments were cloned into the *Eco*RV-site of pUC57. These constructs were purchased from GeneScript, NJ, USA. pUC57_VEGF:HaeIII, pUC57_VEGF:BfaI and phVEGF165.SR were all cleaved by *Bsa*BI and *Bsi*W*I*. The vector was dephosporylated with shrimp alkaline phosphatase from by Roche, Bromma, Sweden and used according to manufacturer's protocol. The fragments were purified on agarose gel and extracted according to the QIAquick protocol by QIAGEN, West Sussex, UK. Fragment and vector were ligated with T4 DNA Ligase.

Mutation for *Bfa*I-site:	**Mutation for *Hae*III-site:**
gtacgatgaactttctgctgtcttg	gtacgatgaactttctgctgtcttg
ggtgcattggagcc'tAgccttgc	ggtgcattggagccttgccttgct
tgctctacctccaccatgccaagt	gctctacctccaccatgccaagt
ggtcccaggctgcacccatggc	ggtcccaggctgcacccatggc
agaaggaggagggcagaatc	agaaggaggagg'Ccagaat
atcacgaagtggtgaagttcatg	catcacgaagtggtgaagttcat
gatgt	ggatgt

Figure 3. Mutated sequence between *Bsi*WI and *Bsa*BI in the hVEGF-gene. Mutation annotated with capital letter. Cleavage site annotated with apostrophe.

[14] Chen (2005), p. 127

2.2 Production of mini-circles

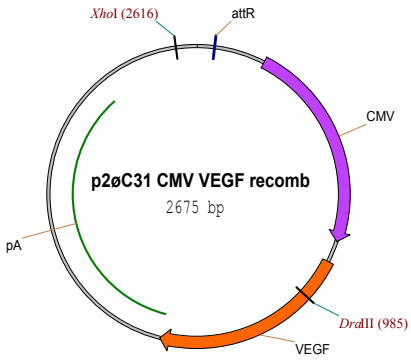

Figure 4. mc_hVEGF: Mini-circle resulting from recombination of p2ΦC31_hVEGF.

2.2.1 Growth of mini-circles

The method used to produce the mini-circles, which can be seen in fig. 4, is based on a protocol kindly provided by Z-Y Chen, Stanford, USA: The p2ΦC31_hVEGF is transformed by heat-shock into *E. coli* strain One Shot Top10 from Invitrogen, Ca, USA. Cells were grown over night in terrific broth (TB) with 50 µg/ml Amp, shaking with 225 rpm at 37˚C. The cells were spun down and resuspended in one-fourth of the original volume in Luria-Bertani broth (LB) pH 7.0 with 1% L-(+)-arabinose. This induces the ΦC31-gene expression. Recombination occurred for two hours at 32˚C. The *I-Sec*I endonuclease was then induced through addition of LB pH 8.0 with 1% L-(+)-arabinose, and the culture was transferred to 37˚C. The bacteria were harvested after two more hours of incubation.

To optimize the production of mini-circles one can elaborate with pH, temperature and medium. The *I-Sec*I endonuclease is known to have an increased activity at higher pH, up to pH 11.[15] We therefore tried several different pH-values. The optimal temperature for the ΦC31-recombinase is 32˚C.[16] The endonuclease on the other hand is known to work efficiently between 30˚C and 45˚C.[17] Growth of the bacteria at 32˚C for all four hours could therefore result in less unrecombined plasmid. To deprive the bacteria of nutrients could cause the bacteria reduce the replication of the unrecombined plasmid, while the enzymes still would be active. In one experiment, we investigated the effect of media composition on the nuclease activity. We grew the bacteria in three different media with pH 7.0, i) Tris with $MgCl_2$ ii) minimal medium M9 iii) LB. Six cultures were grown at 32˚C for four hours and six were transferred to 37˚C after two hours. One medium-type from each temperature-group was resuspended in two thirds of the original volume in medium with pH 8.3. To the other cultures, we added one-eight of the original volume of respective medium with pH 8.3.

2.2.2 Purification of mini-circles

The mini-circles were purified using QIAGEN Plasmid Maxi or Giga Kit according to protocol. In order to favour the mini-circles above the unrecombined p2ΦVEGF and the rest-plasmid, we experimented with elution buffers with different concentrations of salt. We also

[15] Monteilhet (1990) p. 1412
[16] Chen (2003) p. 497
[17] Monteilhet (1990) p. 1410

performed a fractionized collection of the eluate, to 14 fractions. The proportion of mini-circle in the purified samples was quantified using BioRad Quantity One software.

To further cleanse the plasmid from unrecombined p2ΦC31_hVEGF and rest-plasmid, we utilized a DNase, Plasmid-Safe ATP-Dependent DNase from Epicentre, WI, USA. This enzyme hydrolyzes linear double-strand DNA, but does not affect circularized DNA. We treated the samples with *Afl*II, an endonuclease whose restriction site is present only in the rest-plasmid and the unrecombined p2ΦC31_hVEGF. Then the DNase was added. The samples were then purified on a QIAGEN PCR-purification spin column. After this, the mini-circle could be used for transfection of mammal cells *in vitro*.

An alternative method for purifying the mini-circles from the unrecombined plasmid and rest-circle is to separate them based on their different size on a gel-column. This size exclusion chromatography was performed together with Malin Svensson at Vecura, Stockholm, Sweden. A XK 16/70 column (GE Healthcare, Uppsala, Sweden) coupled to a BioCAD 700E (Perseptive biosystems, MA, USA) was packed with Sephacryl S 1000 SF using a two-step method consisting of constant flow (30cm/h) followed by constant pressure (0.7 bar). The packing solution was 0.1 M NaCl. The packed bed was equilibrated with at least two column volumes of running buffer (TE with 0.3M NaCl, pH 7.5) at a flow rate of 20 cm/h followed by sample application. The sample (1ml) was obtained by QIAGEN Giga Kit purification; hence it consisted of a mixture of mini-circle plasmid DNA of 2600 bp, approximately 76%, rest-circle 9700 bp and unrecombined p2ΦC31_hVEG 12000 bp. The DNA was diluted in running buffer to a concentration of 460 μg/ml. The sample was applied and the flow rate was 20 cm/h, the absorbance at 254 nm was monitored and 3 ml fractions were collected.

2.3 Cells and transfection

We used HeLa-cells and HT-1080 cells, grown on Dulbecco's Modified Eagle's Medium supplemented with 10% Foetal Bovine Serum and 100 μg/ml gentamycin (DMEM 10) for the cell-culture studies. In the ELISA-study, the cells were transfected with mc_hVEGF, p2ΦC31_hVEGF, phVEGF165.SR and an EGFPLuc-plasmid kindly provided by Iulian Oprea, Clinical Research Centre, Stockholm, Sweden. The cells were transfected five times with equimolar proportions of plasmid and once with equimass proportions. Three wells in a six-well plate were transfected with each plasmid. In three of the equimolar experiments, the cells were co-transfected with a Gaussia-plasmid for internal reference, kindly provided by Iulian Oprea. Gaussia (hGLuc) is a humanized version of a luciferase from the marine copepod organism *Gaussia Principes*.[18] In the majority of the experiments we used the FuGENE 6 3:1 reagent:DNA-ratio, but we also tried polyethylenimine (PEI).

To test the mutated phVEGF165.SR:HaeIII, cells were co-transfected with this plasmid and the Gaussia-plasmid. Untransfected cells and cells co-transfected with phVEGF165.SR and the Gaussia-plasmid were used as controls.

The cells were harvested 48 hours after transfection. The hVEGF produced by cells in culture is secreted to the medium and easily harvested. The Gaussia is extracted by chemical lysis and repeated freeze-thawing of the cells. RNA from the phVEGF165.SR:HaeIII-transfected cells with controls was prepared using a QIAGEN RNeasy Protect Mini Kit according to the manufacturer's protocol, with the additional DNase digestion steps.

[18] Tannous (2004) p. 436

2.4 Quantification of VEGF-production

2.4.1 ELISA

The amount of hVEGF in the HeLa-cells was measured with an R&D Systems Quantikine Human VEGF sandwich ELISA according to the protocol by R&D systems Inc., MN, USA. The samples were analyzed in duplicates. Gaussia was quantified by an assay where the fluorescence is measured after addition of coelenterazine from BioThema, Handen, Sweden. The samples were measured in triplicates. The Gaussia-value gives an indication of the transfection rate. In the ideal case, the ELISA-value can be divided by the Gaussia-value for the corresponding well, and this will normalize the VEGF-values. However, differences in handling of the samples, pipeting errors and other human factor faults still remains, this makes the quota less reliable. Yet, it can give an indication of the transfection-success in different wells.

2.4.1 Development of the semi-quantitative RT-PCR

The primers for the RT-PCR were constructed with the Primer Express-software and delivered by CyberGene, Huddinge, Sweden. For sequence, see table 1. They were tested on the phVEGF165.SR in standard PCR.

hVEGF-primer fw:	**hVEGF-primer rev:**
GCCTTGTAGAAGCGCGTACG	GGTACTCCTGGAAGATGTCCACC

Table 1. Primers for semi-quantitative RT-PCR of hVEGF, 5'-3' direction.

The resulting fragment is 203 bp. The mutated site for *Bfa*I is at position 53 in the fragment. The site for *Hae*III is at position 122. Hence, digestion of the PCR-fragment from hVEGF:BfaI will result in two fragments of length 53 and 165 bp. hVEGF:HaeIII will be seen as two fragments of 95 and 122 bp.

The hVEGF-cDNA was synthesized according to protocol by Roche with equal amount of RNA from each sample, and the sequence specific reverse primer described in table 1. The cDNA was then used as template in PCR.

In order to determine in which cycles the PCR is still exponential, samples were extracted after each to every second cycle between 20 and 32. Samples from cells transfected with phVEGF165.SR, phVEGF165.SR:HaeIII, and cotransfected with phVEGF165.SR:HaeIII + mc_hVEGF were tested. For the samples transfected with phVEGF165.SR:HaeIII, the PCR fragments were cleaved with *Hae*III. The samples were analyzed on a 1.5 % agarose gel and the band intensity quantified with BioRad Quantity One software.

In the cotransfection with phVEGF165.SR:HaeIII and mc_hVEGF, the cleaved *Hae*III-product was analyzed on a 1.5 % agarose gel and the band intensity quantified with BioRad Quantity One volume rect tool. A smaller fragment will give a weaker signal in a quantified gel although the number of DNA-fragments is the same as in a band consisting of larger fragments. It is therefore necessary to normalize the signal with the size of the fragment, 203 bp for the mc_hVEGF fragment and 165 for the phVEGF165.SR:HaeIII fragment.

3. Results

3.1 Plasmids and cloning

Candidate p2ΦC31_hVEGF-clones were purified and the plasmid transformed into Top 10-cells. The size of produced mini-circles was examined to find the clones where the right fragment had been inserted. In several cases, more than one copy of the insert had been ligated into the vector, resulting in multimer-mini-circles (data not shown). After several attempts, a clone was found where only one hVEGF-fragment had been inserted into p2ΦC31. Compare uncleaved clone 3, white arrow in fig. 5, with e.g. uncleaved clone 5 and 6 where several multimer bands can be seen above the band of correct size, at the black arrows. The resulting plasmid has a size of 11888 bp. The *Dra*III-site is situated in the hVEGF-expression cassette.

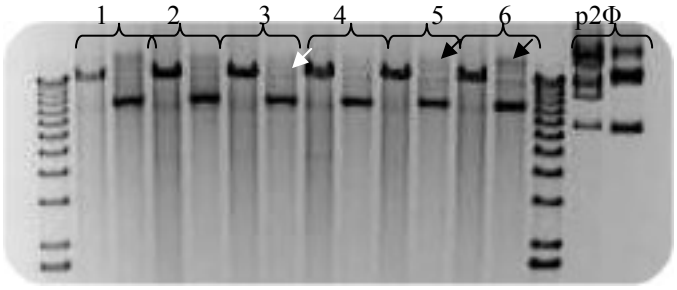

Figure 5. *Dra*III-cleaved resp. uncleaved p2ΦC31_hVEGF -candidates. Candidate 3 has no multimere bands (wite arrow); compare with e.g. candidate 5 and 6 (black arrows). A 1 kb+ size maker was used.

Cloning of the mutated hVEGF-fragments into phVEGF165.SR proved to be quite difficult. For some reason we rarely obtained clones after ligation and transformation, regardless of whether the vector used was dephosphorylated or not. For the phVEGF165.SR:HaeIII-mutant, we eventually got two clones. The hVEGF gene was sequenced, which verified the insertion of the mutated fragment. PCR and HaeIII-cleavage of the PCR-fragment showed that the desired site was present, as can be seen in fig. 6. The phVEGF165.SR:BfaI still remains to be constructed.

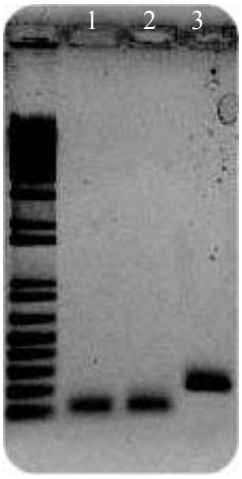

Figure 6. *Hae*III cleaved PCR fragment Samples run on a 1.5% agarose gel. A 1 kb+ size maker was used. Lane 1: phVEGF165.SR:HaeIII clone 1, lane 2: phVEGF165.SR:HaeIII clone 2, lane 3: phVEGF165.SR. Note the size difference between the cleaved mutated fragments (lane 1 and 2) and the unmutated (lane 3). The resolution on a 1.5% agarose gel is not enough to see the two bands resulting from *Hae*III-digestion.

3.1.1 hVEGF expression of mutated plasmid

In order to investigate the function of the phVEGF165.SR:HaeIII, the *in* vitro VEGF-expression of phVEGF165.SR:HaeIII was measured with ELISA and was compared to that of phVEGF165:SR. The cells were co-transfected with Gaussia-plasmid, and the values could be used for normalization. The resulting graph can be seen in fig. 7. It is clear that the mutation did not affect the expression of the protein.

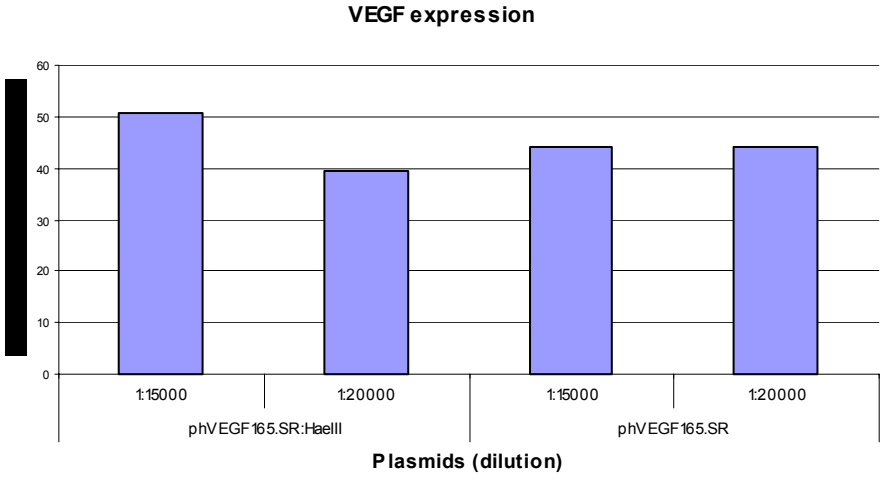

Figure 7. Gaussia-normalized ELISA-values for VEGF-expression in HT 1080-cells that were transfected with equimolar amounts of phVEGF165.SR:HaeIII and phVEGF165.SR.
No significant difference in expression can be seen.

3.2 Production of mini-circles

It should be noted that the ratio of mini-circle vs. unrecombined plasmid and rest circle is much higher in Mini-prep-samples than in the Maxi- and Giga-preps. Somehow, the Mini-prep column retains the large fragments and favours the elution of the smaller mini-circles. This, however, seems not to be the case in large-scale purification.

3.2.1 Growth of mini-circles

3.2.1.1 Effects of pH in growth medium

In order to examine the effect of pH in growth medium on mini-circle production, LB with four different pH-values was used for the endonuclease-activity phase. The results can be seen in fig. 8. It is quite evident that higher pH results in a larger proportion of mini-circle in the purified sample. This is consistent with the fact that the *I-Sec*I endonuclease is known to have an increased activity at higher pH.

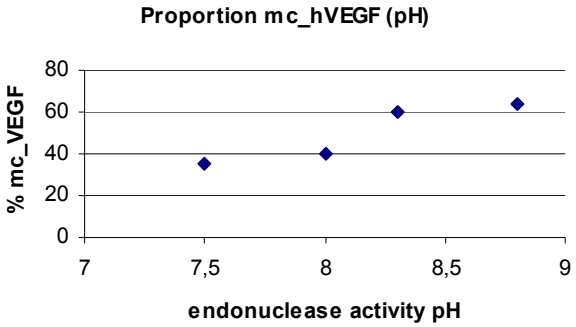

Figure 8. Proportion of mini-circle in purified samples (Maxi-prep) from cultures grown at different pH for endonuclease activity. High pH seems to promote mini-circle production.

3.2.1.2 Effects of incubation time

Due to the supposed degradation of rest-circle and unrecombined plasmid, the incubation time and especially the period of endonuclease activity, should affect the proportion of mini-circle in the sample. This was examined by extracting samples from the culture at different time-points and quantification of the amount of mini-circle in the purified sample. However, as can be seen in fig. 9, the incubation did not affect the proportion of mini-circle in the samples.

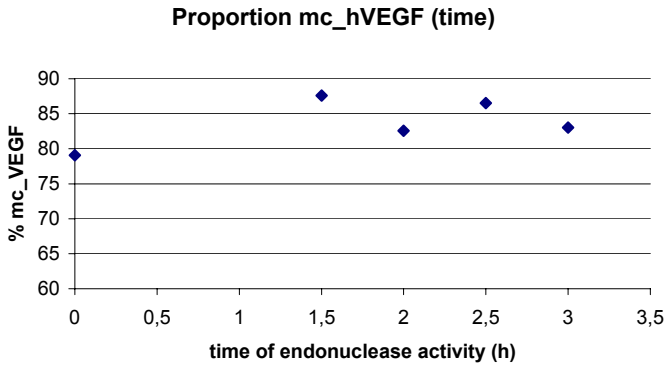

Figure 9. Proportion of mini-circle in purified sample (Mini-prep). Mean values from three cultures. Incubation time appears not to affect the proportion of mini-circle.

3.2.1.3 Effects of growth medium composition and temperature

Results from the assay where the recombination was performed in different media and temperature are presented in table 2 and fig. 10. It is obvious that only bacteria grown in LB will produce significantly large amounts of mini-circle. However, it might be preferable to cultivate the bacteria in 32° C for the entire incubation time. It can be noted in fig. 10 that the samples cultivated in 32° C generally had a higher percentage of mini-circle. This temperature promotes the ΦC31-integrase activity.

Sample#	Medium	pH	T (deg C)	% mc_hVEGF
1	LB	7.0 + 8.3	32	82
2	LB + M9	7.0 + 8.3	32	61
3	LB + Tris $MgCl_2$	7.0 + 8.3	32	67
4	LB	8.3	32	80
5	M9	8.3	32	57
6	Tris $MgCl_2$	8.3	32	50
7	LB	7.0 + 8.3	37	67
8	LB + M9	7.0 + 8.3	37	63
9	LB + Tris $MgCl_2$	7.0 + 8.3	37	62
10	LB	8.3	37	68
11	M9	8.3	37	58
12	Tris $MgCl_2$	8.3	37	48

Table 2. Percentage of mini-circle in purified samples (Mini-prep) from cultures grown in different media and temperature for recombination and endonucleases activity. Mini-circle formation is best in the samples where the bacteria were grown in rich media, LB.

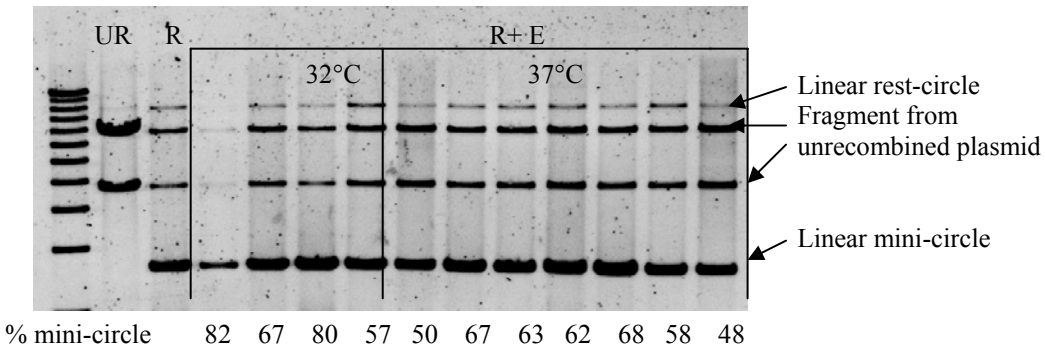

Figure 10. Gel picture from the assay where the bacteria were cultured in different media and temperature. The Mini-prep-samples have been cut with *Xho*I and *Afl*II. A 1 kb+ size maker was used. An incubation temperature of 32°C promotes mini-circle formation.

Eventually we rationalized the process to adding 1/5 of the culture volume of LB ph 7.0 with 14µl 5M NaOH/ml after two hours of incubation at 32˚C. This resulted in a pH of ca 8.1. The bacteria were harvested after an additional two hours growth in 32˚C.

3.2.1.4 Functionality of the *I-Sec*I site

In fig. 10, it is obvious that the samples are contaminated primarily by unrecombined plasmid, although the proportion of undigested rest-circle cannot be neglected. This raised the question whether the p2ΦC31_hVEGF-preparation contains a fraction of plasmid with a defective *I-Sec*I site. Purified sample was cleaved with *I-Sec*I according to protocol. The results, seen in fig. 11 confirm that the p2ΦC31_hVEGF-preparation has a functional *I-Sec*I site.

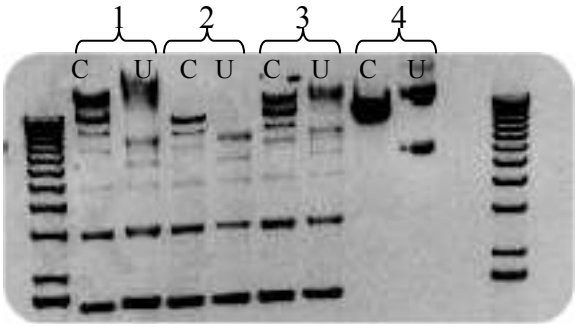

Figure 11. C: Sample cleaved with *I-Sec*I, U: Uncleaved sample. Sample 1 and 3: Maxi-prep mini-circle sample, sample 2: Mini-prep mini-circle sample, sample 4: p2ΦC31. A 1 kb+ size marker was used. In all samples, the rest-circle and the unrecombined plasmids have an intact *I-Sec*I site

3.2.2 Purification of mini-circles

3.2.2.1 Eluation in QIAGEN preparations

As has been stated earlier, Mini-preps produced a higher percentage of mini-circles. Therefore, we tried different salt concentrations in the eluation buffer for Maxi-prep in order to see if we could mimic the retention of larger fragments. However, changes in the salt concentration of the elution buffer did not seem to promote elution of mini circle over larger plasmids. In the fractionized collection of eluate, the sample was mostly present in fraction four to seven, as can be seen in fig. 12. The concentration of mini-circle was slightly higher in the fraction six and seven; 76% compared to 71% in fraction number three and four.

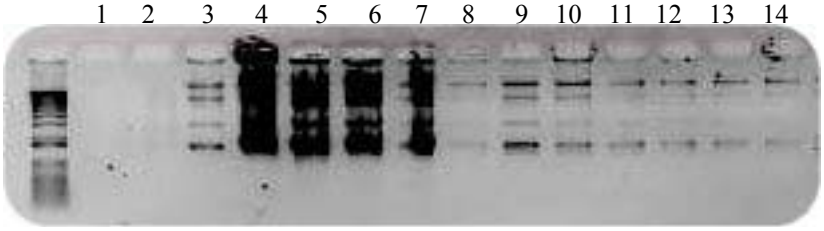

Figure 12. Fractionized collection of mc_hVEGF from Maxi-prep purification. A 1 kb+ size maker was used.

3.2.2.2 DNase treatment

It is evident from e.g. fig. 10 that the in culture digestion of unrecombined plasmid and rest-circle is not sufficient, additional purification was needed. In order to degrade the rest-circle and unrecombined p2ΦC31_hVEGF still present in the Maxi-prep samples, we utilized a DNase that only affects linearized DNA. Fig. 13 shows a maxi-prep purified sample after linearizing of unrecombined p2ΦC31_hVEGF and rest-circle and digestion with Plasmid-Safe ATP-Dependent DNase. The DNase clearly degrades the linear DNA-strands, and it seems to leave the circular mini-plasmid unharmed. After digestion, there is only mini-circle, super-coiled and nicked, in the sample. Possibly, there is also a fraction of dimer mini-circle, although TE-treatment to see open circular plasmid indicates that there is only one plasmid form. However, in order to use the samples for e.g. transfection, it must be cleaned from enzymes and salts. Purification on a QIAGEN PCR-purification spin colon was sufficient, but there was a substantial loss of plasmid.

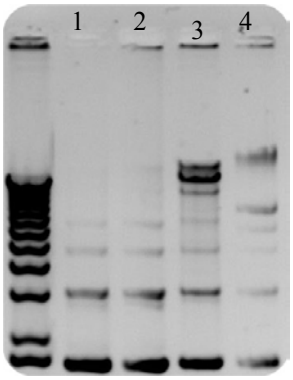

Figure 13. Mini-circle sample from Maxi-prep cleaved with *Afl*II after various treatments: Lane 1: DNase treated and PCR-purified, lane 2: DNase treated, lane 3: untreated, lane 4: uncleaved. A 1 kb+ size maker was used. Note the loss of rest-circle and unrecombined plasmid in lane 1 and 2 compared to lane 3.

3.2.2.3 Size exclusion chromatography

As the DNase treatment is costly and causes a loss of plasmid, it is necessary to develop other purification methods. In the Sephacryl size exclusion chromatography, the packed bed height was 68 cm, the reduced plate height (HEPT/d_p) was 4.9 and the asymmetry factor (A_s) was 1.2. 460 µg Giga-prep purified mini-circle was loaded onto the column. Two peaks eluted from the column, one at 58 ml containing approximately 80 µg DNA and one at 82 ml containing approximately 250 µg DNA. The chromatogram can be seen in Appendix II. Fractions were collected and analysed using agarose gel electrophoresis; see fig. 14. The first peak contained the larger parental DNA of 11888 bp whereas the mini circle DNA of 2675 bp was found in the second peak.

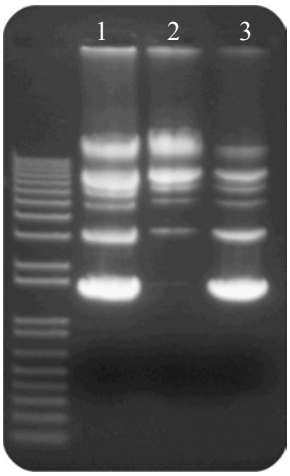

Figure 14. Samples from size exclusion chromatography. Lane 1: mini-circle from Giga-prep sample, lane 2: 1st peak, lane 3: 2nd peak. A 1 kb+ size maker was used. The first peak contains no mini-circle, and the second peak significantly less rest-circle and unrecombined plasmid.

3.3 Quantification of VEGF production

3.3.1 ELISA

The *in vitro* hVEGF-expression from the three different plasmids, phVEGF165.SR, mc_hVEGF and p2ΦC31_hVEGF, was measured with ELISA. The transformation with the

EGFP-plasmid showed that the transfections were successful, though in the early experiments, somewhat weak. In most transfection assays where the cells were co-transfected with the Gaussia-plasmid, all that could be concluded was that the transfection rate was overall similar in all wells. Two experiments with equimolar amount of plasmid failed due to weak transfection. In two of the equimolar transfections, the mc_hVEGF-value was about the same as the phVEGF165.SR-value; no prominent difference between the two plasmids could be detected when simply looking at the ELISA-values. However, in the experiment where the Gaussia-value could be used for correction, the mini-circle value was significantly higher; about four times the phVEGF165.SR-value.This causes the large standard deviation in the mini-circle values in fig. 15.

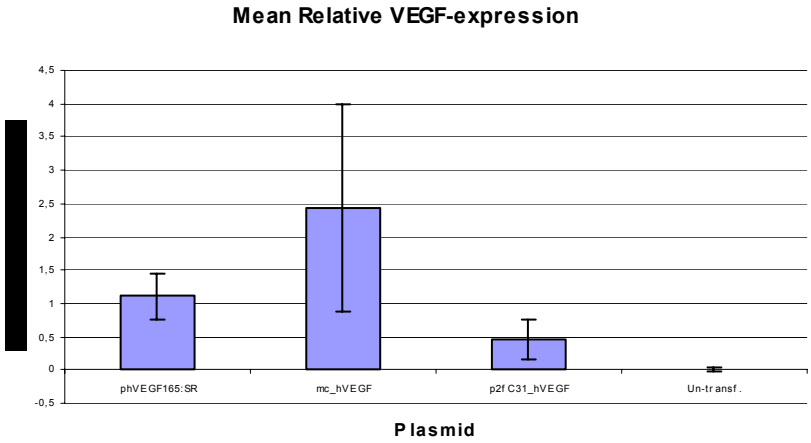

Figure 15. Relative VEGF-expression. The values were divided with the phVEGF165.SR-value using the dilution with the lowest standard deviation in the ELISA-doubles.

3.3.2 Development of the semi-quantitative RT-PCR

The specificity of the constructed primers was first tested on phVEGF165.SR in standard PCR. As can be seen in fig. 16, there are no unspecific binding and replication. Lane four shows that there was hardly any problem with primer-dimer formation.

Figure 16. Product from PCR with the primers shown in table 1. Lane 1: phVEGF165.SR, lane 2: phVEGF165.SR:HaeIII clone 1, lane 3: phVEGF165.SR:HaeIII clone 2, lane 4: Negative control. A 1 kb+ size maker was used.

The primers were then tested in end-point RT-PCR with cDNA synthesized from whole RNA extracted according to the QIAGEN-protocol. End-point RT-PCR product was cleaved with *Hae*III and run on a 1.5% agarose gel, see fig. 17. The expected fragment sizes of 204 respective 122 bp shows that RNA is an adequate template for the cDNA synthesis.

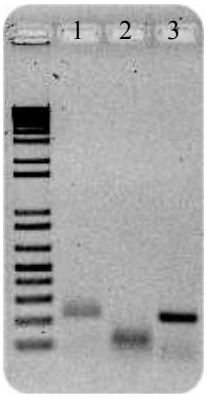

Figure 17. *Hae*III-cleaved RT-PCR products. Lane 1: cDNA from phVEGF165.SR, lane 2: cDNA from phVEGF165.SR:HaeIII, lane 3: Positive control phVEGF165.SR. A 1 kb+ size maker was used. Note the size difference between the cleaved mutated fragment (lane 2) and the non-mutated (lane 1 and 3)

To perform a semi-quantitative PCR it is important to stop the reaction while it is still in the exponential phase. As can be seen in fig. 18, the PCR with hVEGF-cDNA from cells cotransfected with phVEGF165.SR:HaeIII and mc_hVEGF is still exponential in cycle 29. This graph is representative for the all the experiments testing the number of PCR-cycles. We decided to run the semi-quantitative RT-PCR up to and including cycle 26 making sure the PCR will still be in the exponential phase. The full program can be seen in the Appendix I. When the quantified gel electrophoresis band signals from the cotransfection experiment of phVEGF165.SR:HaeIII and mc_hVEGF were normalized to fragment size, the hVEGF mRNA level from the mini-circle is 1.3 times higher than that of the larger plasmid.

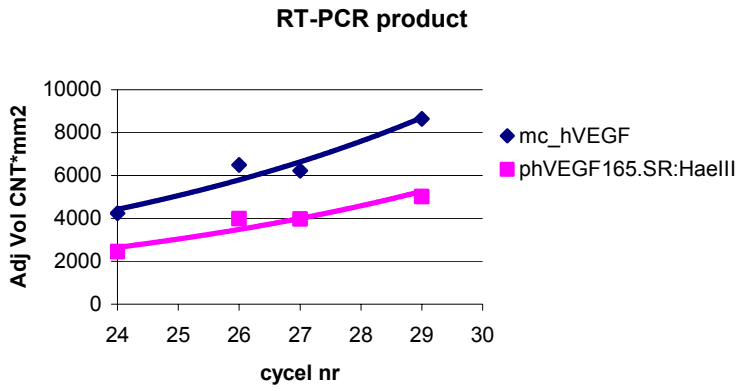

Figure 18. Quantification of HaeIII-cleaved RT-PCR product from cells cotransfected with phVEGF165.SR:HaeIII and mc_hVEGF

4. Discussion

The purpose of this master thesis was to produce and test a small plasmid consisting only of the expression cassette for hVEGF, as well as to develop a method for measuring hVEGF-mRNA-levels in cells cotransfected with mc_hVEGF and phVEGF165.SR. The results indicate that the mini-circle plasmid has an equal, if not higher, expression of hVEGF compared to a larger plasmid.

4.1 Mini-circle production

We can observe a high percentage of undigested rest circle and unrecombined parental plasmid in the QIAGEN purified samples; unfortunately the in culture degradation is not wholly efficient. It is possible that we, by chance, selected a clone whose *I-Sec*I endonuclease is somehow less active. This would explain why the rest-circle is not digested to further extent. Sequencing of the *I-Sec*I-gene of the p2ΦC31_hVEGF-clone would determine if this is the case. Higher pH during the endonuclease phase seems to increase the fraction of mini-circle in the samples. However, it is unclear if longer incubation time increases the amount of mini-circle or merely results in more unrecombined plasmid. This is peculiar, as one would assume that a longer incubation time would result in a more thorough degradation of rest-circle and unrecombined p2ΦC31_hVEGF.

In the assay where several types of media and different incubation temperature were tried, it is evident that depriving the bacteria of nutritious media will not induce higher mini-circle production. As can be seen in fig. 10, an incubation temperature of 32˚C for both recombination and endonuclease activity might be favourable, as it enhances the ΦC31-integrase activity However, the medium-assay clearly shows the importance of keeping the growing bacteria well nourished and prosperous; a lower incubation temperature might deteriorate the health of the bacteria. This is also true for the pH of the growth medium.

As a way to cleanse the samples of the unrecombined plasmid and rest-circle still contaminating the QIAGEN purified samples, a DNase was utilized. It is clear that the Plasmid-Safe ATP-Dependent DNase degraded only the linearized p2ΦC31_hVEGF and rest-circle, and left the circular mini-plasmid unharmed. In this way, we obtained enough pure mini-circle for the transfection assays. However, this method of purification is hardly suitable for large-scale production. Not only is the DNase expensive, it is also necessary to purify the samples further in order to get rid of the enzyme and salts. The size exclusion chromatography is a much more promising course of action. As an additional purification, the samples could be run on a Plasmid Select column, which separates nicked plasmid from super-coiled. In large-scale production, a purification method that crude bacteria-lysate as start-material would be preferred, as QIAGEN purification in large scale will be impractical.

4.2 VEGF expression in mc_hVEGF compared to phVEGF195.SR

It cannot be said with certainty that there is any significant difference in the hVEGF-expression of phVEGF165.SR and mc_hVEGF. The Gaussia-corrected values indicated that the mini-circle has a four-fold higher expression than the phVEGF165.SR-plasmid, but more experiments are needed to confirm this. However, in order to conclude if the mini-circle is less prone to gene silencing that the phVEGF165.SR, *in vivo* studies must be performed where the expression is followed for several weeks.

The phVEGF165.SR is a plasmid known for its high VEGF-expression. It contains some sequences that could seem unnecessary, e.g. the Kan-resistance gene is inserted into an untranscribed Amp-resistance gene. The mini-circle, on the other hand, is a stricter, less complex plasmid devoid of bacterial sequences. The fewer antibiotic-resistance-genes spread the better; this speaks for the mini-circle. When considering what vector to use for gene-therapy treatment, the mini-circle is an appealing alternative even if the expression is not significantly better than that of the phVEGF165.SR.

It is interesting to note that neither in the equimass transfection experiment could any significant increase of the mc_hVEGF-expression be detected. Considering that the mini-circle is about half the size of the phVEGF165.SR, hence the cells were transfected with almost the double amount of mini-circle. This experiment was however only performed once, and without Gaussia-correction. It could be interesting to repeat the equimass transfection, and this time with Gaussia.

4.3 The semi-quantitative RT-PCR-method

To be able to distinguish between mRNA from mc_hVEGF and phVEGF165.SR, two mutated versions of the gene were constructed. The mutations are silent, with a shift in the third base of a leucine-codon or glycin-codon respectively: CTT to CTA and GGG to GGC. When doing this one must consider how common the codons in question are in the organism.[19] This is important as usage of rare-codons might affect the translation from mRNA to protein. The mutated hVEGF should preferably act as the normal hVEGF, the only difference being one base.

*Hae*III is an enzyme that has full activity in PCR-buffer. Hence, it is not necessary to purify the samples after PCR. *Bfa*I has unfortunately no activity in PCR-buffer according to Roche Diagnostics and New England Biolabs. The PCR-product must therefore be purified before cleavage with *Bfa*I. This is easily done with e.g. a QIAquick PCR purification kit. However, in these purifications some material is lost. This is a disadvantage as the resulting samples are to be used for semi-quantitative PCR. It is important that the amount of purified sample is proportional to the amount of template in the PCR reaction. It might have been better to choose two enzymes that have a 100% activity in PCR-buffer, so that the intermediate cleaning-step could have been avoided. As the assay is constructed today, both samples will have to be purified, as it is important to treat the samples the same way.

In the cotransfection experiment with mc_hVEGF and phVEGF165.SR:HaeIII, the mini-circle has a slightly higher mRNA level. This value is comparable to the non-Gaussia-corrected ELISA-values. However, this experiment was only performed once. A problem lies in the fact that the mini-circle fragment is of the same size as uncleaved phVEGF165.SR:HaeIII. Although the *Hae*III-cleavage is believed to be nearly complete, which can be seen in control-digestion, the experimental setup is not optimal. This problem will however not occur when both mutated versions of hVEGF are available.

4.4 Mini-circle systems

The system for a *Streptomyces* temperate phage integrase ΦC31-mediated recombination enables the creation of small plasmids consisting of a given sequence which is introduced

[19] Ikemura T. (1985) Table 5

between the recombination sites *att A* and *attB*. This enables us to create plasmids devoid of bacterial sequences in the bacteria. This system, which is used in this thesis, was however not the first mini-circle system to be developed. Bigger *et al.*[20] constructed a system for mini-circles based on a bacterial *Cre* recombinase expression system. *Cre* induces recombination between *loxP*-sites. The Bigger mini-circle system utilizes an arabinose control of the *Cre*-gene, much like the Chen system. However, the gene for the recombinase is not situated in a plasmid but mutated into the DNA of the bacterial strain. This is an additional mutation step not necessary in the Chen system where the recombinase is encoded by the plasmid that will parent the mini-circle. However, this results in a rather large parental plasmid. Quite recently, Bigger *et al.* developed a new bacterial strain for *Cre* mini-circle production.[21] Another difference between the Bigger system and the Chen system is that the *loxP*-sites are not destroyed after recombination; hence the plasmids can recombine again, resulting in multimers of mini-circles or restoration of the parental plasmid. The Bigger-group has developed a mutated *loxP*-site which induces a shift in kinetics towards mini-circle production. In the Bigger system, the rest-circle and unrecombined circle is not destroyed in the bacteria, hence the mini-circle must be purified by linearization of the unwanted plasmids and a cesium chloride gradient. Bigger *et al.* detected a higher gene expression in HeLa cells transfected with mini-circle plasmid compared to cells transfected with the parental plasmid or the plasmid carrying the original expression cassette.

An alternative method developed by Riu *et al.* at the same group as Mark Kay at Stanford produces a transgene expression cassette devoid of bacterial sequences *in vivo* by cotransfecting cells with one plasmid containing the expression cassette of the gene of interest between *I-Sec*I-sites and one carrying the *I-Sec*I-gene[22]. In this system, when the endonuclease gene is expressed, the expression cassette carrying the gene in interest will be cut from the bacterial sequences, and able to form mini-circles or concatamers. The bacterial rest-circle will be silenced. A similar system described in the same article uses an FLP-mediated recombination to form mini-circles *in vivo*. These are interesting system, and have been shown to increase the transient gene expression. It does not have the problems with purification that have been discussed in this thesis. However, the bacterial sequences will be present in the transfected cells, as will the selection marker used. One of the beauties with the Chen mini-circle system is that the antibiotic resistance gene will not be transferred to the target cell. Another disadvantage of the Riu system is necessity of the cotransfection of the two plasmids; if only one plasmid is introduced to the cell, the system does not function. There is no need of an assisting plasmid in the Chen system.

In order for this system to create plasmids that have a persistent gene-expression, we must understand what features in the bacterial DNA induce silencing. Chen[23] suggests that features of the bacterial sequences induce histone modification, which in turn gives rise to formation of condensed chromatin, similar to endogenous heterochromatin. As Chen shows[24], it is the covalent linkage between the expression cassette and the bacterial sequences that induces gene silencing. This implicates that the chromatin condensation spreads from the bacterial DNA into the gene. It is therefore important to make sure that the expression cassette cloned into in p2ΦC31 is not flanked by any bacterial sequences.

[20] Bigger (2001)
[21] Tolmachov (2006)
[22] Riu (2005) p. 558 fw.
[23] Chen (2004) p. 862-863
[24] Ibid. p. 857-860

4.5 Other systems

There are other possible ways to produce a plasmid devoid of antibiotics resistance genes and sequences that trigger inflammatory responses.

As the unmethylated CpG-motifs are thought to be highly immunogenic, one might prefer to use sequences where these motifs are rare. There are CpG-free plasmids systems available, e.g. from Invitrogen. In their construct, all the elements required for replication and selection of the plasmid in *E. coli* and gene expression in mammalian cells are completely devoid of CpG dinucleotides. Another possible way to evade the inflammatory response has been developed by Liu and coworkers. In their system, the DNA is codelivered with inflammatory suppressors that inhibit cytokine production once the complex has been delivered to the immune cells. [25]

Systems that does not relay on antibiotics as a selective marker are desirable as there will be no spreading of genes for antibiotics resistance; the plasmid contains none. One such system have been developed by Hägg *et al.* They have deleted the chromosomal copy of the essential gene *infA*, which encodes translation initiation factor 1 (IF1). IF1 is a small protein involved in the initiation of translation in prokaryotes and it is essential for cell viability. The gene is present in the plasmid instead. According to Hägg *et al.*, the vector was stable for at least 120 generations in the absence of antibiotics.[26] This system can be used without any restriction regarding growth media or temperature.

Soubrier *et al.* have developed a different strategy to avoid antibiotic resistance genes. Their selectable marker is a sequence encoding a bacterial suppressor tRNA. The expressed tRNA allows the correct translation of the mRNA from a mutated chromosomal *argE*-gene. Thereby it allows the bacteria to prosper in minimal media lacking arginine[27]. An additional feature in the Soubrier plasmid is the conditional origin of replication; it is only functional in a specifically engineered *E. coli* strain. This ensures that the plasmid will not spread and grow in other bacteria or in treated patients.

Cranenburgh *et al.* argue that a plasmid containing a coding gene as selection marker will be a metabolic burden to the cell, due to the constitutive expression of the gene. A non-expressing selection element is to be preferred. Their system is based on selection by operator-repressor titration (ORT). An essential chromosomal gene is placed under negative control by a repressor protein, and a copy of the repressor gene is introduced into the bacterial DNA. When the repressor protein is expressed, it binds to the negative promoter and the essential gene is silenced; the bacteria die. When a multicopy plasmid carrying the same promoter sequence is present in the bacteria, the plasmids binding the repressor protein depress the chromosomal operator and allow the expression of the essential gene. Hence, the bacteria prosper. Cranenburgh *et al.* have showed that the plasmid does not necessarily have to be a high-copy plasmid.[28] The essential gene in question could be a gene for antibiotics resistance as it would not be the plasmid that contained the antibiotics resistance gene. However, as an antibiotic free system is to be preferred, Cranenburgh *et al*. have developed an ORT system for the *dapD* gene controlled by a *lac* operator sequence.[29]

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[25] Liu (2004)
[26] Hägg (2004) p. 28
[27] Soubrier (1999) p. 1483
[28] Cranenburgh (2004)
[29] Cranenburgh (2001)

One disadvantage with these selection systems, as with the Bigger mini-circle system, is that it will only function in the corresponding bacterial strain. However, the benefits of being independent of antibiotics and resistance genes cannot be neglected. In order to produce a plasmid that is stable in the bacteria in the absence of antibiotics and is devoid of bacterial sequences, which induces not only inflammatory response but also gene silencing once inside the nucleus, a mini-circle system based on e.g. the *infA* selectivity marker is an appealing thought. It could easily be combined with the Chen mini-circles system. This would result in a vector for gene therapy produced in an antibiotics-free environment and devoid of bacterial sequences. As a mini-circle contains no bacterial origin of replication sequences, there is no risk of spreading of the vector into other bacteria or treated patients.

4.6 And what now?

The mini-circle needs to be tested in a co-transfection assay with phVEGF165.SR.Therefore the mutated version of a mini-circle must be constructed, as well as the second mutated version of hVEGF. The entire mutated expression cassette of phVEGF165.SR will be PCR-cloned, with addition of *Pfu*I and *Spe*I-sites. The cassette will be ligated into a WGB-pUC57 that contains sites for binding of peptide nucleic acid (PNA). PNA is used for targeting of plasmids, e.g. with nucleic location signals for enhanced uptake of plasmid. The phVEGF165.SR-expression cassette will be cloned into the WGP-pUC57 in front of the PNA-site. From this plasmid, the whole fragment can be cleaved with *Xho*I and *Spe*I and ligated between these sites in p2ΦC31. With these two mutated versions of hVEGF-plasmids, a co-transfection of human cell lines can be performed *in vitro*. The resulting mRNA can be extracted and quantified with the semi-quantitative RT-PCR method developed in this thesis.

Considering the problem with PCR purification discussed above, it is evident that the development of probes for a Q-RT-PCR is of great importance. Not only would it increase the sensitivity of the method but also reduce the number of steps, as neither purification nor restriction enzymes and gel-electrophoresis would be necessary. Less handling of samples is usually better. I suggest further introduction of silent mutations in the region flanked by the existing primers. This would create two variants that could be distinguished by two allelic probes for Q-PCR.

4.7 Concluding remarks

In this thesis, I constructed a plasmid that can form a hVEGF-encoding mini-circle plasmid. These mini-circles are devoid of bacterial sequences and antibiotic resistance genes. They have an equal if not higher *in vitro* VEGF expression compared to that of an existing larger plasmid that is known for its high expression. In order to optimize mini-circle production, different growth conditions and purification methods have been tested. A Sephacryl size exclusion chromatography seems to be the best alternative for large-scale purification. To construct the ideal vector for gene therapy, it must be verified that the mini-circle is not silenced *in vivo*, and the selective marker should be replaced with an antibiotic-independent system. The semi-quantitative PCR developed in this thesis will aid in the future *in vivo* experiments, and be the basis for an even more sensitive Q-RT-PCR.

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Databases:

- Codon Usage Database: http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=Homo+sapiens+[gbpri] (13th September 2005)

Appendix I: PCR programs

Ordinary PCR:

97˚C 6’
94˚C 30’’
53,6˚C 30’ } 30 cycles
72˚C 30’’
72˚C 10’
8˚C forever

Semi-quantitative RT-PCR:

cDNA syntesis:
25˚C 10 min
42˚C 60 min
99˚C 5 min
4˚C forever

PCR:
97˚C 6’
94˚C 30’’
53,6˚C 30’ } 26 cycles
72˚C 30’’
72˚C 10’
8˚C forever

Appendix II: Chromatogram

From the size exclusion chromatography with Giga-prep purified mini-circle sample.