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DIPLOMARBEIT

The Influence of the -1639 G>A Promoter Variant on the Expression of the Vitamin K Epoxide Reductase Gene in HepG2 Cells

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TABLE OF CONTENTS

i. Acknowledgements

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ii. Index of Abbreviations

bp base pairs
cDNA complementary DNA
Cys cysteine residue
c-fw common forward
ddH_2O double distilled water
DMSO dimethyl sulfoxide
DNA desoxy ribonucleic acid
dNTP desoxy ribonucleotide
DTT dithiothreitol
dsDNA doublestrand DNA
EDTA ethylenediaminetetraacetic acid
ER endoplasmic reticulum
F factor
FBS fetal bovine serum
fw forward
GAPDH glyceraldehyd-3-phosphate dehydrogenase
GGCX gamma glutamyl carboxylase
Gla gamma carboxyglutamate/ γ-carboxyglutamic acid
Glu glutamate residue/ glutamic acid
kb kilo bases
kDa kilo Dalton
KH2 vitamin K hydroquinone
KO vitamin K epoxide
MCS multiple cloning site
MM Mastermix
nt nucleotide
OD optical density
PCR polymerase chain reaction
PDI protein disulfide isomerase
RDA recommended daily allowance
rev reverse
RLU relative luminescence unit

RNA ribonucleic acid

RNAP RNA polymerase

RT reverse transcriptase/ room temperature

rpm rounds per minute

siRNA short interfering ribonucleic acid

SNP single nucleotide polymorphism

TBE Tris Boric Acid EDTA

TFBS transcription factor binding site

TE Tris EDTA

TF transcription factor

TM transmembrane

TSS transcription start site

UTR untranslated region

VK vitamin K

VKCFD combined deficiency of vitamin K-dependent clotting factors

VKD vitamin K dependent

VKOR vitamin K epoxide reductase

VKORC1 vitamin K epoxide reductase complex subunit 1

WR warfarin resistance

I. ABSTRACT

Vitamin K epoxide reductase complex subunit 1 (VKORC1) recycles vitamin K epoxide to reduced vitamin K in the process of the components of the vitamin K cycle. Reduced vitamin K is used as a co-factor for the γ-carboxylation of glutamic acid residues in vitamin K-dependent proteins. These proteins are only active following gamma-carboxylation. VKORC1 is the rate-limiting factor of vitamin K recycling and thus of gamma-carboxylation.

Several polymorphisms in the VKORC1 gene have been identified and proposed to have an influence on the expression of the gene. One of these genetic variants is the promoter polymorphism -1639G>A. The G allele is said to be associated with a significantly higher promoter activity and higher expression of mRNA. Additionally, the A allele in homozygous form is associated with increased warfarin-sensitivity.

Since there have also been reports that the SNP -1639G>A does not have an influence on the expression of the VKORC1 gene, it was the objective of this study to conduct experiments to re-evaluate the influence of the promoter variation on the expression of the VKOR gene.
Two VKORC1 promoter constructs that were available in the laboratory, pGL3-G and pGL3-A, were used for the experiments. pGL3-G contained the G nucleotide at the position -1639, pGL3-A contained the A nucleotide. The constructs were transfected into HepG2 cells and promoter activity was measured in a Dual Luciferase Reporter Assay.

Two different transfection methods were evaluated and compared. While the commonly used reagent Lipofectamine produced rather variable and unreliable results the newly developed Metafectene PRO yielded more reproducible results and higher values in the Luciferase Assay.

The Luciferase Assay with cells transfected with Metafectene PRO indicated only a minor difference in the promoter activity between the two variants, with G giving slightly higher values.

In conclusion, expression of the reporter gene was not significantly influenced by the G or the A version of the promoter in HepG2 cells. However, there is still the possibility that the influence of the SNP -1639G>A is tissue-specific.

II. ZUSAMMENFASSUNG

Vitamin K Epoxid Reduktase Komplex Untereinheit 1 (VKORC1) regeneriert im Vitamin K Zyklus Vitamin K Epoxid zu reduziertem Vitamin K. Die reduzierte Form von Vitamin K wirkt als Kofaktor bei der Gamma-Carboxylierung der Vitamin K abhängigen Proteine. Diese Proteine werden erst durch die Gamma-Carboxylierung in ihre aktive Form übergeführt. VKORC1 ist dabei der limitierende Faktor der Regenerationsrate von reduziertem Vitamin K und damit auch der Carboxylierung.

Einige Polymorphismen, die im VKORC1 Gen identifiziert wurden, dürften einen wesentlichen Einfluß auf die Expression des Gens ausüben. Zu den biologisch relevanten Varianten gehört unter anderem der Polymorphismus -1639G>A, der in der Promoterregion von VKORC1 lokalisiert ist. Befindet sich das Nukleotid G an der Position -1639, so ist die Aktivität des Promoters angeblich signifikant höher, als in Anwesenheit des Nukleotids A an Position -1639. Aufgrund früherer Publikationen dürfte auch die Expression von mRNA in Gegenwart der G Variante erhöht sein.
Es wurde außerdem festgestellt, dass homozygote Träger des A Allels sensitiver auf das Antikoagulant Warfarin reagieren.

Andere Quellen berichten, dass der Polymorphismus -1639G>A keinen Einfluss auf die Expression des VKORC1 Gens ausübt. Es war daher das Ziel dieser Arbeit, den Einfluss des Polymorphismus in eigenständigen Experimenten zu untersuchen und zu bewerten.
Dazu wurden die beiden im Labor zur Verfügung stehenden Konstrukte pGL3-G und pGL3-A verwendet. pGL3-G trug die Promotervariante -1639G, pGL3-A trug hingegen -1639A. Die Konstrukte wurden in HepG2 Zellen transfiziert und die Promoteraktivität anschließend mittels eines Dual Luciferase Reporter Assays ermittelt.

Es wurden zwei unterschiedliche Transfektionsmethoden evaluiert. Im Vergleich erzielte das häufig angewandte Reagenz Lipofectamine sehr variable und unzuverlässige Ergebnisse. Das neu entwickelte Metafectene PRO hingegen ergab im Luciferase Assay viel höhere Werte, zeigte jedoch auch eine relativ hohe Schwankungsbreite der Resultate, die allerdings nicht so hoch war wie mit Lipofectamine.

Die Ergebnisse, die im Luciferase Assay mit den mit Metafectene PRO transfizierten Zellen erzielt wurden, zeigten nur einen minimalen Unterschied zwischen den beiden Promotervarianten, wobei das Konstrukt pGL3-G geringfügig höhere Promoteraktivität aufwies. Die Expression des Reporter-Gens wurde nicht signifikant durch eine der beiden Promotervarianten beeinflusst. Es besteht allerdings immer noch die Möglichkeit, dass der Polymorphismus -1639G>A die Expression von VKORC1 gewebsabhängig reguliert.

III. INTRODUCTION

1. Vitamin K Epoxide Reductase Complex Subunit 1 (VKORC1)

1.1. Identification of the VKORC1 Gene

VKOR stands for vitamin K epoxide reductase. This name was first introduced in 1970 by Bell *et al*.[2] to describe an enzyme that was found to play an active part in the vitamin K cycle. It catalyzes the regeneration of vitamin K epoxide to vitamin K.

Although the enzyme was identified in 1970, for a long time scientists were neither able to purify the VKOR protein nor to identify the gene expressing it.

In 2002 Fregin *et al*. found out that humans with a hereditary deficiency in several vitamin-K-dependent clotting factors have disorders in VKOR activity that can be mapped to human chromosome 16.[13]

Subsequently Rost *et al*. (2004) focused their research on chromosome 16 in patients and rats with inherited defects of VKOR activity to localize the gene coding for VKOR. They found one particular gene that showed mutations in two families with defective vitamin-K-dependent clotting factors and in four families with hereditary warfarin resistance. Additionally they identified similar genes in fish, frogs and even mosquitoes.

The candidate gene encoded a small transmembrane protein that is found in the endoplasmatic reticulum. Since they were not sure if this protein was only a component of a larger complex, they named it vitamin K epoxide reductase complex subunit 1 or in short VKORC1.[33]

At the same time the research team of Darrel W. Stafford also succeeded in identifying the gene coding for VKOR by conducting siRNA knock-down experiments of 13 possible candidate genes encoding transmembrane proteins within the region 16p12-q21.[20] They were the first group to identify

an unknown gene in mammalian cells through siRNA. Expression of the gene was subsequently confirmed in insect cells.

1.2. VKORC1 Gene

The gene VKORC1 is located on the short arm of the human chromosome 16 in the region 16p11.2 and contains 5126 base pairs. It includes 3 exons with exon 1 spanning 173 bp, exon 2 encompassing 110 bp and exon 3 spanning 209 bp which in total amounts to a coding region of 492 bp. There are two introns with intron 1 spanning 1125 bp and intron 2 spanning 1969 bp.
The gene also includes a 5' untranslated region that stretches over 226 bp and a 3' untranslated region that extends over 295 bp.

VKORC1 encodes a protein of the same name that is made up of 163 amino acids and has a molecular mass of 18 kDa. It is a putative endoplasmic reticulum protein with three transmembrane domains.
Stafford and his team (2004) were able to identify VKORC1 not only in humans but also in rodents. In mice the gene is located on chromosome 7F3, in rats on chromosome 1.
Interestingly, the human genome contains several DNA segments in other regions that are partially identical to VKORC1. In particular there is one segment on the X chromosome that is 93% identical and one on chromosome 1 that is 91% identical to the VKORC1 sequence. Yet, these segments do not code for a protein similar to VKORC1 and are considered pseudogenes.[34]

Both Stafford's and Oldenburg's research teams found a homologous protein in the genome of the African malaria mosquito *Anopheles gambiae* but not in *Drosophila melanogaster* even though both species encode mostly the same conserved genes[53] and despite *D.melanogaster* expressing a homolog of vitamin-K-dependent gamma-glutamyl carboxylase.[21]
In 2004 Hugh M. Robertson of the University of Illinois undertook a phylogenetic analysis and finally found a VKORC1 homolog in Drosophila

as well as in the sea urchin *Strongylocentrotus purpuratus* and the urochordate sea squirts *Ciona intestinalis* and *Ciona savignyi*.[31]
Homologs of the VKORC1 gene are also present in plants, protists, archea, and bacteria.[26]
Furthermore in the human genome a paralog of VKORC1 is present on chromosome 7 that was designated VKORC1-like1 (VKORC1L1) by Rost *et al*. (2004). This gene and protein is even more conserved in its sequence across the vertebrates than VKORC1 which differs by 8% in rats and mice, indicating that the VKORC1L1 sequence shows a very high degree of identity across species, above all in humans, rats and mice. Yet, the function of VKORC1L1 is still unclear.
According to Robertson's (2004) analysis the vertebrate lineage of VKORC1 is monophyletic and its rapid divergence could indicate that its function in vitamin K recycling is a derived one and VKORC1L1 might be the key to the original function.

The function of VKORC1 as it is known at present is that of a co-factor in the vitamin-K-cycle. Since the intake of vitamin K in the human diet is limited, the reduced form vitamin K epoxide that is generated during the gamma carboxylation reaction must be re-converted to vitamin K, so that it can be used again during gamma carboxylation. The enzyme VKORC1 is essential for the reduction of vitamin K.

1.3. VKORC1 Structure and Topology

The enzyme expressed by the VKORC1 gene is a 163-amino acid integral membrane protein located in the ER. Since the structure and function of proteins are interdependent, it is necessary to define the membrane topology of VKORC1 in order to fully understand its function and activity.

In 2005 the topology of the VKORC1 protein was experimentally elucidated by Tie *et al*.[43] They used seven common computer programs to predict the topology and afterwards conducted *in vitro* experiments to confirm the results. Results of both analyses suggested that VKORC1 is a type III membrane protein with three transmembrane (TM) domains and

the orientation of Nexoplasmic/ Ccytoplasmic. Another example for a type III membrane protein would be cytochrome P450.

The N terminus lies in the lumen of the ER while the C terminus resides in the cytoplasm. The three TM domains span amino acids 10-29, 101-123, and 127-149.

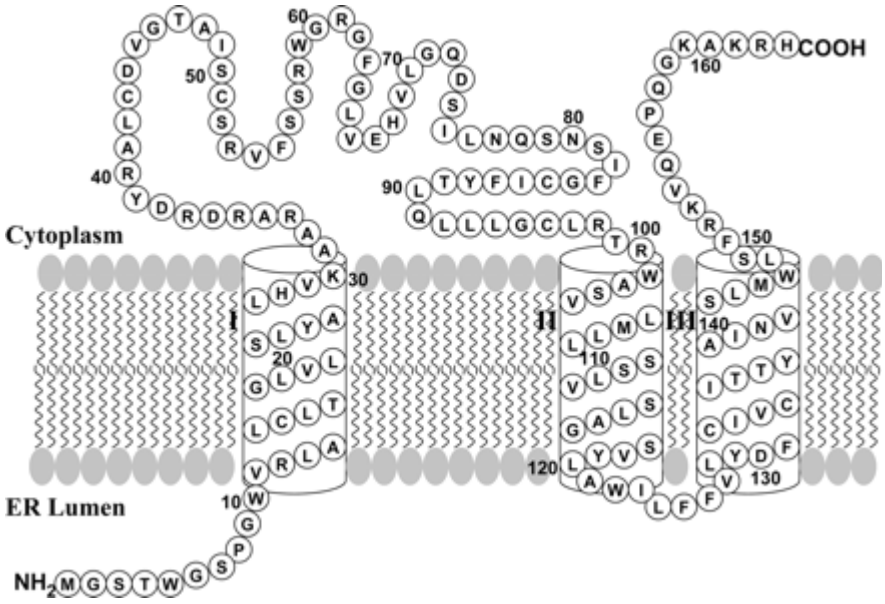


Fig.1: Proposed membrane topology of VKORC1.[43]

As depicted in Fig.1, only 12 amino acids are located in the lumen, while the majority lies in the cytoplasm in the form of a big loop that stretches from amino acid 31 to 100, and the C terminus which includes amino acids 148 to 163. The remaining 66 amino acids make up the three TM domains which are all α-helices in structure.

Considering the very short N terminal domain, it is highly unlikely that the active site residues are located in the lumen. There is a much higher probability of the active site is residing either in the cytoplasm or within the membrane. The latter hypothesis is supported by the fact that vitamin K is very hydrophobic.

In 2004 Goodstadt and Ponting[16] proposed that a CXXC motif forms the active site of VKORC1, as this is the case in a number of other oxidoreductases, such as thioredoxin. If the CXXC motif is the active site

in VKOR, it would be located in the hydrophobic environment of the third TM domain and include Cys-132 and Cys-135, two of the four highly conserved cysteines in VKOR, the other two being Cys-43 and Cys-51. This proposal is consistent with the observation of Wajih *et al*. that the mutation of Cys-132 or Cys-135 to alanine abolishes VKOR activity.[46]

Since the discovery of the VKORC1 protein in 1970 [2] numerous attempts to purify the molecule have failed. After having analyzed its structure and topology, Tie *et al*.[43] suggested that these difficulties in purification were due to the localization of the active motif inside a TM segment. Solubilisation of the membrane prior to purification of the protein leads to an immediate loss of function. Thus the protein cannot be detected.

In 2006 Chu *et al*. of the research team of Darrel W. Stafford finally succeeded in purifying the VKORC1 protein.[6]

1.4. VKORC1 Function and Mechanism of Action

The function of VKORC1 is to regenerate vitamin K and very likely also vitamin K hydroquinone (KH_2) from vitamin K 2,3–epoxide (KO). KH_2 is an essential co-factor for the posttranslational carboxylation of glutamic acid residues (Glu) to γ-carboxy glutamic acid (Gla). Vitamin K-dependent proteins (VKD) are posttranslationally modified by this reaction and can only fulfil their biological functions after complete gamma-carboxylation. These biological functions are coagulation, signalling and bone metabolism.

C-132 and C-135 of the redox motif are essential for the conversion of vitamin K epoxide to vitamin K and of vitamin K to vitamin K hydroquinone.[18]

Since at present no three-dimensional structural data is available for VKORC1, only hypotheses on how its mechanism for the reduction of vitamin K epoxide by VKORC1 works have been published. The most probable hypothesis is that of Davis *et al*.[11] which represents a modification of the Silverman mechanism[37]. It is proposed that to start the reaction mechanism, a reducing agent is necessary which provides electrons. This task is assumed to be the responsibility of thiol reagents,

presumably thioredoxin[37], since it has been observed in some plants and bacteria that the VKORC1 active site is fused to thioredoxin-like (Trx) domains[11], which, like VKORC1, contain CXXC motifs.
In contrast, Wajih *et al*. (2007) hypothesized that the reaction is driven by protein disulfide isomerase (PDI) together with the folding of proteins within the ER.[47]

Whichever agent might be responsible for the reduction, it is currently believed that this reduction of the CXXC redox center leads to the breakage of the disulfide bonds assumed to link the Cys residues in the active center. The disulfide is reduced to sulfhydryl.
The second important step is the reduction of vitamin K epoxide to the quinone form by protonation. A cysteine attack follows, leading to the loss of the proton and rearrangement followed by another protonation, attack by the second cysteine and finally loss of a water molecule by reductive elimination.
Davis *et al*.[11] assumed the Cys residues to be CH_3SH and the corresponding disulfide CH_3SSCH_3. The starting point hereby is CH_3S^- and the overall chemical reaction in short is:
$HSCH_3 + {}^-SCH_3 + H^+ \rightarrow CH_3SSCH_3 + H_2O$.

Wang *et al*. (2005) observed that the VKOR protein is expressed ubiquitously in the human body, but primarily in the liver, kidney, heart, and skeletal muscle. They found expression to be higher in ventricular aneurysm tissue, adult heart after myocardial infarction and in fetal heart.
This group hypothesized that VKOR might be involved in angiogenesis as well as tumor development and growth.[50]

1.5. Polymorphisms in the VKORC1 Gene

Polymorphisms, the short term for single nucleotide polymorphisms or, further abbreviated, SNPs, are point mutations that occur frequently in the population. Generally, genetic variants that occur with a frequency of at least 1% studying a given population, qualify as polymorphism. Since the identification of the VKORC1 gene, several SNPs have been identified and

the number is still increasing with every large study. Most of these SNPs are located in untranslated regions.

In 2005 Geisen *et al*. conducted a study among 200 blood donors from Western Germany and published a list of all SNPs that they found within the genomic sequence of VKORC1 including 1.8kb of the 5'- and 1.5kb of the 3'-flanking region (Table 1). They identified a total of 28 different SNPs. Three of these are located in the coding region, but they are silent and do not change the structure of the protein. The other 25 SNPs are located in non coding regions of the VKORC1 gene: seven in the promoter, 11 in introns and 5 in the 3'-UTR. In 14 SNPs the frequency of the minor allele is at least 1%, the other half of the polymorphisms was rare in the study cohort.[15]

Nucleotide position VKORC1		Nucleotide exchange	Gene region	Allele frequency	
				1 (%)	2 (%)
-1795		T>C	Promoter	99,75	0,25
-1639		G>A	Promoter	58,5	41,5
-1453		G>A	Promoter	98,75	1,25
-1272		A>C	Promoter	99,75	0,25
-941		C>T	Promoter	99,5	0,5
-311		T>C	Promoter	99,75	0,25
-292		T>C	Promoter	99,5	0,5
36		G>A	Exon 1	98,75	1,25
129		C>T	Exon 1	99	1
181		C>T	Intron 1	99,75	0,25
261		C>T	Intron 1	99,75	0,25
305		A>G	Intron 1	99,75	0,25
497		T>G	Intron 1	69,75	30,25
503		G>A	Intron 1	99,75	0,25
556		G>A	Intron 1	99,75	0,25
698		C>T	Intron 1	79,75	20,25
1173		C>T	Intron 1	58,5	41,5
1542		G>C	Intron 2	58,5	41,5
2255		C>T	Intron 2	58,5	41,5
2714		A>G	Intron 2	90,75	9,25
3462		C>T	Exon 3	99,5	0,5
3647		G>A	3`Region	99,75	0,25
3730		G>A	3`Region	61,75	38,25
4182		G>A	3`Region	99,75	0,25
4799		A>G	3`Region	99	1
5155		C>T	3`Region	98,75	1,25
5192		G>A	3`Region	99,25	0,75
5198		G>A	3`Region	99,75	0,25

Table 1: Polymorphisms in the VKORC1 region detected in 200 blood donors from Germany. [15]

As mentioned above, new polymorphisms have been reported for VKORC1. Therefore, the list shown can by no means be considered complete. Up to now more than 40 different SNPs have been identified.
In 2004 Rost *et al*. identified two genetic variants in VKORC1 which are associated with phenotypic effects.
The first phenotype is combined deficiency of vitamin K dependent coagulation factors type 2, or abbreviated VKCFD2. The second one is resistance to coumarin-type anticoagulant drugs or warfarin resistance (WR). Warfarin is an important oral anticoagulant used for the treatment of patients that have already suffered a thromboembolic event or who are at high risk of suffering such an event. Warfarin is the most popular and most commonly used coumarin-type anticoagulant drug worldwide.
It has been shown that response to warfarin treatment differs between individuals. One underlying cause are mutations in the VKORC1 gene. WR is primarily caused by the heterozygously inherited mutation 416 A>G in Intron 1.[33] In contrast, VKCFD2 is the result of a homozygous point mutation, 292 C>T, in exon 3 in the patients studied,

Rieder *et al*. (2005) determined 5 specific SNPs that are clearly associated with variability in warfarin dose requirement in European Americans. These SNPs are: -4931T>C, -1639G>A, 1173C>T, 1542G>C, and 2255C>T. They define 2 major haplotype groups designated A and B. Haplotype A carries the minor alleles and is associated with lower mRNA expression of VKORC1 and the need for a lower warfarin maintenance dose. Haplotype B carries the major alleles, shows higher mRNA expression and has higher warfarin dose requirement.[29] Dose requirement means that patients require individually different doses of the warfarin to prevent thromboembolic events.

2. Vitamin K and the Vitamin K Cycle

2.1. Vitamin K

Vitamin K is a fat-soluble vitamin that occurs naturally in two main forms, designated vitamin K_1 and K_2. Both are derivatives of quinone which is a common constituent of biologically relevant molecules.

Vitamin K_1 is also known as phylloquinone, which is synthesized by plants and is contained in green leafy vegetables like spinach and broccoli. Vitamin K_2 is the collective name for a whole range of vitamin K forms found in foods like liver, milk, cheese, and fermented soy products, like Natto.[55] They are menaquinones, designated MK-n, with n standing for the number of isoprenoid residues in the aliphatic side chain of the molecule. In food, the most important menaquinones are MK-7, MK-8 and MK-9. Schurgers *et al*. (2007) reported that MK-7 is the most effective form of vitamin K in the organism because it has a much longer half-life time in the circulation and a much higher cofactor activity in the vitamin K cycle than any other K vitamin.[35]

All forms of vitamin K are similar in structure and are collectively called 2-methyl-1,4-naphthoquinones (Fig.2).

It has been observed that vitamin K_1 is primarily taken up in the liver while vitamin K_2 accumulates in the arteries and extra-hepatic locations.[39]

Studies have shown that nutrition contributes mainly vitamin K_1. This is because menaquinones are only contained in very small amounts of just a few µg per 100g in the foods mentioned above.[36] This is not enough to meet the RDA, according to the National Research Council 1989, of 1µg/kg•d (per kg body weight per day).[3]

Newer data show that the ideal amount of vitamin K would be 100µg/d. Newer data report that MK-7 also contributes significantly to human vitamin K levels taken up by nutrients. Cheese is supposed to contain 50-75mg of MK-7 per 100g.[35]

The K in vitamin K stands for the Danish word “Koagulation”, referring to the process of blood clotting. The name was given by Henrik Dam in the 1920’s after he found that chickens fed a fat-free diet developed a bleeding diathesis, but no known factor could be made responsible for this. Therefore he named the missing compound vitamin K and subsequently received, together with Edward Doisy, the nobel price in medicine or physiology for his discovery.[55] Today it is clear that vitamin K is essential for the proper function of several blood coagulation factors. Lack of vitamin K can lead to internal haemorrhaging and bleeding disorders. It’s most important biological function is that of a cofactor in the γ-carboxylation of proteins involved in the coagulation cascade and the formation of blood clots. The γ-carboxylation is a step necessary for the activation of a number of important proteins including coagulation proteins, which are therefore referred to as “vitamin K-dependent”.

The carboxylation reaction is catalysed by the enzyme vitamin-K-dependent carboxylase which uses oxygen and a reduced form of vitamin K to add a molecule of carbon dioxide to glutamic acid, thereby producing γ-carboxyglutamic acid. This modification allows clotting factors to bind calcium ions, associate with membrane surfaces and clot blood.[55] The second product that is generated during catalysis is vitamin K 2,3-epoxide which is recycled to reduced vitamin K by vitamin K epoxide reductase (VKOR).

The enzyme that is responsible for this modification is the vitamin K gamma glutamyl carboxylase (GGCX). It carboxylates the glutamic acid residue (Glu) of the protein, which is then referred to as the Gla amino acid (Gla). All reactions together are called the vitamin K cycle (Fig.2).[40]

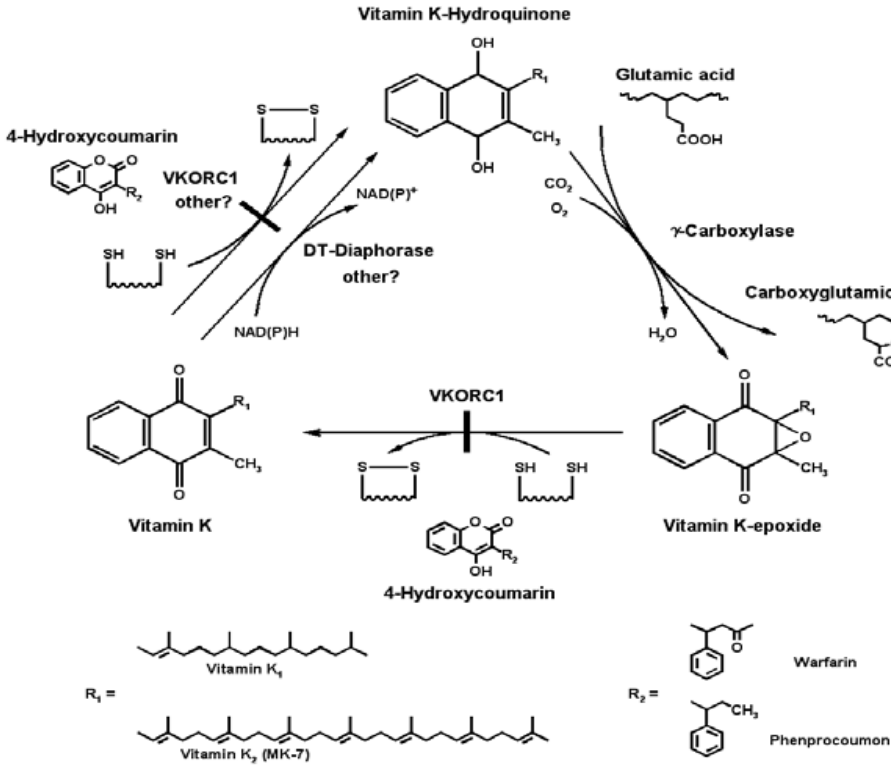


Fig.2: The Vitamin K cycle.[26]

The vitamin K cycle can be divided into three main reactions:
Carboxylation by GGCX
Regeneration of K from KO by VKORC1
Conversion of K to KH2
Which enzyme this last reaction depends on is not yet clear. It might either be catalyzed by VKORC1 or by a seperate enzyme, i.e. the DT diaphorase (NAD(P)H dehydrogenase).[40]

The γ-carboxylase requires not only reduced vitamin K as an obligate cofactor but also CO_2 and O_2.[41]
The cycle starts with the binding of the carboxylase to its substrate, a VKD protein, which has a carboxylation recognition site within its mature protein sequence. This sequence is part of the propeptide which binds to GGCX and is defined by a ZFZXXXXA motif, with Z being an aliphatic hydrophobic residue like Ile, Val or Leu, F being phenylalanin, A being alanine and X standing for any amino acid.[14]

The carboxylase is a so-called processive protein, which means the enzyme binds tightly to its substrate and all carboxylations for this one substrate occur in one single binding event. During the reaction the γ-proton on glutamic acid is removed and CO_2 is added to the remaining γ-carbon. In the course of this process vitamin K hydroquinone binds the mentioned hydrogen from the γ-carbon which leads to the conversion of KH_2 to KO.[14]

Dowd *et al.* (1995) proposed that this happens because KH2 acts as a strong base that substracts a proton from the glutamates in the VKD proteins to make carboxylation possible.[12] For every molecule of Gla that is generated in the vitamin K cycle, one molecule of KO is produced.
The rate of carboxylation depends on the level of available vitamin K.[40] This amount is limited since the dietary intake of vitamin K is usually relatively low. It is therefore the main responsibility of the vitamin K cycle to regenerate vitamin K for reuse to guarantee the efficiency of carboxylation which is absolutely necessary for keeping the VKD proteins and the whole coagulation system in order.
Since VKORC1 recycles vitamin K, VKORC1 is the rate-limiting factor of carboxylation.

2.2. VKD Proteins

VKD proteins include proteins involved in haemostasis, coagulation factors VII, IX, X, prothrombin (procoagulant proteins), protein Z, protein S and protein C (anticoagulant proteins). These proteins contain 10-12 γ-carboxyglutamic acid residues. The second group of VKD proteins comprises proteins of mineralized tissues. These include osteocalcin (bone Gla protein) and matrix Gla protein.
Another VKD protein is Gas 6 which functions as a ligand for the Axl family of tyrosine kinase receptors[19] and potentiates the growth of smooth muscle cells.[14] Also counted among the VKD proteins are four putative transmembrane proteins, PRGP1 and PRGP2, named so for their proline rich protein characteristic[14], TmG3, and TmG4.[46]

Another group of known VKD proteins is that of conantokins which are conotoxins. These are small, paralyzing neuroactive peptides that are injected into prey by cone snail harpoons.[14]

γ-glutamyl carboxylase itself can also be considered a VKD protein since its function depends on vitamin K as a cofactor.[14]
GGCX is, like VKORC1, an integral TM protein located in the rough ER.[5] It is 758 amino acids long, has five TM domains and a molecular weight of about 94 kDa. Its NH_2 terminus is located in the cytoplasm, the carboxy terminus lies inside the lumen.[40] The human carboxylase gene is located on chromosome 2 at position 2p12. It is 13 kb long and contains 15 exons.[14] The carboxylase gene has been shown to be conserved not only in vertebrates like humans and zebrafish, but also in chordates such as *Ciona intestinalis* and a number of invertebrates like *Drosophila melanogaster* and *Conus* (cone snail).[54]
The biological function of the γ-glutamyl carboxylase is the activation of VKD proteins by carboxylation. Together with carboxylation the carboxylase confers metal binding properties on its substrates. These are very important for protein-membrane interaction.[38]
Through metal binding properties calcium ions are drawn to the plasma proteins and stimulate structural changes in which phospholipide binding sites are exposed of the hydrophobic residues on the surface of the Gla domain.[14] The phosholipide binding sites for instance allow the blood coagulation factors to bind to the membranes of cells they react with.

Functional deficiencies of all vitamin K dependent coagulation factors or other VKD proteins leading to diseases, like hereditary combined VKD coagulation factor deficiency, can either be caused by mutations in the VKORC1 gene or, very rarely, by mutations in the γ-glutamyl carboxylase gene.[10]
Patients who show such deficiencies are usually administered vitamin K. If this treatment is not effective, it is very likely that the γ-carboxylase is the impaired component.[5]

2.3. Vitamin K Antagonists

The substances that specifically inhibit VKORC1 function are the so-called VK antagonists which are derivatives of 4-hydroxycoumarin, or in short coumarin. These include acenocoumarol and warfarin, the latter being the most popular VK antagonist and anticoagulant.[35]

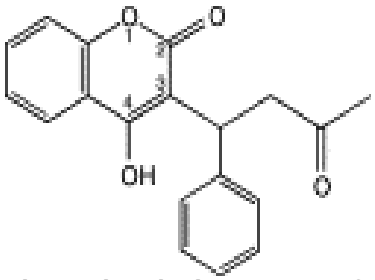


Fig.3: Chemical Structure of Warfarin *[53]*

Warfarin (Fig.3) is a medication used for oral treatment and prevention of thromboembolic events because it “thins” blood. It is in fact the most widely used and prescribed anticoagulant worldwide and has been in use since 1955 when US-President Dwight Eisenhower received warfarin as medical treatment after a heart attack.

Its discovery dates back to the early 1940s. Before that time cows had been bleeding to death in the northern United States after eating mouldy sweet clover hay. In 1940 Karl Link discovered that the responsible fungal product could only be a vitamin K antagonist and named it warfarin, after the Wisconsin Alumni Research Foundation, to which he assigned the patent rights.[33]

Warfarin causes intense internal bleeding when administered in large doses and is therefore also used as a very effective rat poison. In small doses it can, however, prevent lethal blood clots that can form in the lungs, hearts and brains of susceptible people, and thus save lives.

It has been observed that some rats and mice develop resistance to warfarin and similar poisons, a condition that can also occur in humans. Resistance to warfarin in the human context does not mean that the person's system shows no reaction to the medication at all. However,

extraordinarily high doses of warfarin will be necessary to antagonize blood clotting.[33]

It is estimated that warfarin prevents twenty stroke events per induced bleeding episode. Still, warfarin is under-used since dosage control is rather complicated because patients respond quite differently to the treatment and there is always the risk of inducing bleeding.[34]
Warfarin acts as an antagonist to VKORC1 and thus to the carboxylation of VKD proteins. It is now believed that SNPs in VKORC1 have a high influence on the dose response to the medication.
Rost *et al*. (2005) assume that the target for warfarin is the redox center in VKORC1 (Fig.4), to which warfarin binds and thereby prevents carboxylation and subsequently coagulation.[32] Different SNPs might have different effects on this reaction.

Indeed, doses of warfarin causing adequate anticoagulation are correlated with SNPs in the genes for VKORC1 and cytochrome P450 2C9 (CYP2C9).[7] In 2004, Ansell *et al*. published guidelines suggesting initial warfarin dosing of 5 to 10mg per day for people receiving first treatment.[1] However, experience has shown that some patients need a much lower or much higher dose. Incorrect dosing can lead to bleeding in high responders or to thrombosis in low responding individuals. Warfarin sensitive people need doses of around 1.5 mg/d. As has been shown, polymorphisms in VKORC1 have an effect on the formation of reduced vitamin K which in turn affects warfarin dose response and blood clotting.[49]

For instance D'Andrea *et al*. (2005) found that people carrying the T allele of the polymorphism 1173T>C in intron 1 require a lower dose of warfarin than those carrying the C allele. This indicates a lower activity of the VKORC1 1173 T allele to revert KO to KH_2.[9]
Montes *et al*. (2006) found that the A allele of the -1639G>A promoter variant is associated with a low dose requirement for the anticoagulant acenocoumarol.[23]

Other polymorphisms result in a high dose requirement and some mutations can also cause warfarin resistance such as the heterozygous missense mutation 416A>G discovered by Rost *et al*.[33]

3. Promoters and Promoter Variants

3.1. Promoters

Promoters are DNA regions located upstream of genes. They belong to the non-coding part of the DNA strand in the 5'-untranslated regions and contain certain elements, the so-called transcription factor binding sites (TBFS), transcription factors (TF) can bind to. Promoters are highly conserved motifs with a length of 6-12bp and represent regulatory sequence elements that are recognized by the TF.[27]

Every gene has its own promoter where transcription for this one particular gene is started and regulated. Transcription starts downstream of the promoter and proceeds in the direction 5'-3', resulting in a newly synthesized mRNA strand having the orientation 3'-5'.[25] Promoters are always located on the sense strand of the DNA – which is transcribed; the antisense strand does not contain a promoter.

Promoters act as transcription start sites (TSS), which means that the process of transcription begins with the RNA polymerase (RNAP) binding to particular DNA segments within the promoter. The TSS is characterized by very short consensus sequences of 6-10bp. This short sequence principally opens the possibility of the RNAP to bind to similar sequences somewhere else in the gene. However, usually the RNAP locates the right binding site. What guarantees the specificity is still not quite clear.[28]

In prokaryotic genes the RNAP is able to initiate transcription by itself, under the condition that the σ-factor is present. In eukaryotic genes the RNAP needs transcription factors that bind to their recognition sequences within the promoter and constitute the transcription-initiation-complex together with the polymerase. The TF are responsible for phosphorylation

and subsequent release of the RNAP which then starts the transcription process.[25]

Promoters of eukaryotic as well as of prokarytic genes contain characteristic consensus sequences that are essential for the correct binding of the RNAP to the DNA strand. In prokaryotic or bacterial promoters these sequences are the Pribnow- or TATA-Box or -10-region, because the nt at the starting point is designated +1, with the sequence 5'-TATAAT-3' located 10bp upstream of the TSS and the -35-region located 35bp upstream of +1 with the sequence 5'-TTGACA-3'. These elements do not need to have the exact same sequence as pictured here and need not be identical in every gene but have to be similar, for example the TATA-Box can also have the sequence 5'-TATGAT-3' and can still function as binding site.

Often a third element can be found that is called UP-element. The abbreviation UP results from the element's location directly UPstream of the -35-region. It contains a number of AT basepairs which are recognized by the α-subunit of the RNAP that binds to this particular region of the promoter.

Eukaryotic promoters also contain the TATA-Box but this one is located at -25 base pairs upstream. Its sequence is 5'-TATAAAT-3' and is recognized and bound to by the RNAPII which catalyzes the synthesis of the mRNA strand in eukaryotes. The second important sequence is the CAAT-Box that is located at about position -70 and has the sequence 5'-CAAT-3'.[51]

Especially in eukaryotic cells the vast majority of genes is usually silent and is not transcribed until a certain gene product is needed. The mechanism of turning off genes is called silencing. The process is carried out by proteins called silencers. The counter mechanism is called enhancing and is carried out by so-called enhancers. A process somewhere in the middle between these two is the up- and down-regulation of the gene expression which is influenced by the TFs. All three groups of proteins need *cis*-acting regulatory elements or sequences, that lie promoter-proximal, to bind to. The promoter-proximal elements in eukaryotes are the same as the UP-elements in prokaryotes. They are

found within about 100-200bp upstream of +1. The CAAT-Box is one of these elements, as well as a GC-rich segment that is often found a little further upstream.[17]

3.2. The Promoter Variant -1639G>A in the VKORC1 Gene

Promoter mutations are point mutations within the promoter of a gene. They can lead to an increased or decreased expression of a particular gene. These point mutations can change binding sites for proteins. If a binding site for a protein is changed that normally activates transcription, gene expression decreases.[25]

In 2005, Yuan *et al*. conducted a study to determine the effect of the -1639G>A promoter polymorphism in VKORC1 on warfarin sensitivity and promoter activity.[52] The group found that patients carrying the -1639A allele in homozygous form (A/A) were warfarin sensitive (<1.5mg/d) and had low dose requirements. The authors hypothesized that this was because the -1639G>A SNP is located in an E-Box with the consensus sequence CA/GNNTG. E-Box sites are important elements for mediating cell and tissue type specific transcription, like in muscle, neuron, liver, and pancreas.[52] In carriers of the -1639A allele the consensus sequence of the E-Box is CANNTG, in carriers of the wild type allele -1639G the second base is changed and the sequence is converted to CGNNTG. Yuan *et al*. conducted a promoter assay in HepG2 cells which showed that the CGNNTG sequence increases the promoter activity by 44%.[52]

Another study by Bodin *et al*. (2005) could not confirm these results. Although they used the same cell line as Yuan *et al*. (2005), no difference in promoter activity between the -1639A and G alleles could be observed.[4] One possible explanation for this difference in results might be that Yuan *et al*. used lipofectamin for transfection whereas Bodin *et al*. used calcium phosphate. Also, the promoter sequence that was used for both studies differed by a few base pairs.

A very recent study by Wang *et al*. (2008) again confirmed that the G allele increases promoter activity. These authors also tested mRNA expression in a reporter gene assay using HepG2 cells and found that the -1639A allele is associated with a 2-fold lower level of VKORC1 mRNA and therefore also reduced warfarin maintenance dose. But they also observed that the effect of the SNP is tissue-dependent, because they could not reproduce the results obtained in HepG2 cells in B lymphocytes and heart tissue.[48]

4. Full-Length cDNA Constructs

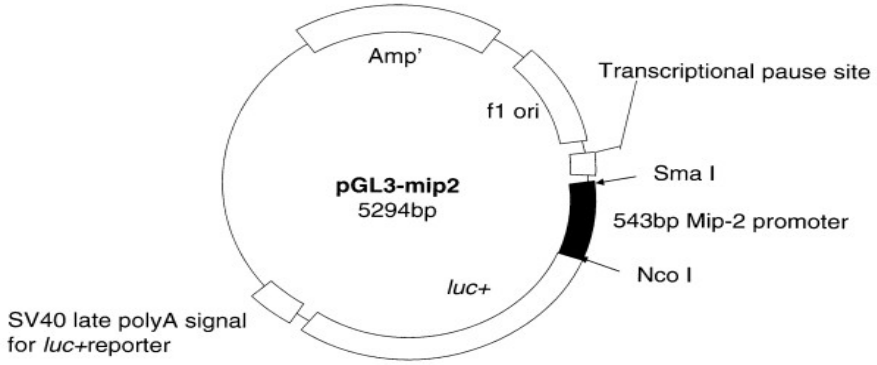


Fig.4. Example for a full-length construct: pGL3 basic vector with mip2 gene insert[60]

cDNA constructs comprise the sequences of the coding region from the CAP site to the poly A site. The template for the cDNA is the coding sequence of the mRNA.

The first step of constructing a full-length cDNA construct is the isolation of total RNA from cell material. Relatively high amounts of mRNA are required and should be at µg level.[22] The second step is synthesis of cDNA, a process that is accomplished by reverse transcriptase which is responsible for creating the complementary DNA strands to the single mRNA strands.

After synthesis the full-length cDNA is enriched by PCR amplification with sequence specific primers which should also add restriction sites to the sequence, which will later be necessary for cleavage with restriction enzymes.

To generate a transferrable cDNA construct, cloning into a suitable vector is required. The vector has to have the capacity of taking up fragments of the length of the construct. For smaller inserts of a size up to a few kb, plasmid vectors are suitable.
Both, vector and insert have to be digested with restriction enzymes to create matching ends. Restriction enzyme digestion also linearizes the vector and thus enables it to take up the insert DNA.
The insert is integrated into the vector during the process of ligation which is accomplished by an enzyme called ligase. The ligase joins the ends of the two DNA fragments, namely vector and insert, and produces the full-length construct.
Subsequently the construct can be transformed in bacterial cells. The full-length constructs that have been obtained can now be, for instance, transfected into cells for further experiments, such as testing expression levels in certain cell lines.

5. Focus of this study

This diploma thesis focuses on the gene variant -1639G>A in the promoter region of the VKORC1 gene.

In the first part of the diploma thesis the -1639G and -1639A variant were examined in a cell culture model to find out if they indeed influence the expression of the VKORC1 gene. As pointed out earlier, the current literature on this topic is controversial.
As it could not be excluded that the transfection methods contribute to or influence the results of the reporter gene assays and the interpretation of the effect of the promoter sequence on gene expression, different transfection methods should be evaluated.

In the second part of the thesis, a construct of the VKORC1 promoter and coding region was prepared and cloned.

IV. MATERIALS AND METHODS

1. Materials

1.1. Reagents

Agarose

Sigma Aldrich, Germany

AmpliTaq Gold Polymerase and 10x PCR Buffer

Roche, USA

Aqua bidestillata/ ddH_2O

Mayrhofer Pharmazeutika, Austria

Bacteriological Agar

Sigma Aldrich, Germany

Big Dye® Terminator v3.1 Cycle Sequencing Kit, Big Dye® Terminator Mix, 5x Sequencing Buffer

Applied Bioscience, UK

Criterion™ 5% Acrylamid Precast Gel

Bio-Rad Laboratories, USA

Dimethyl Sulfoxide (DMSO)

Sigma Aldrich, Germany

DMEM+GlutaMAX™-I 1x

Gibco, UK

Dual Luciferase® Reporter Assay System

Promega Corporation, USA

Ethidium bromide (10mg/ ml)

Sigma Aldrich, Germany

FastRuler™ DNA Ladder, High Range

Fermentas International, Canada

Fetal Bovine Serum (FBS)

Gibco, UK

Gel Star

Invitrogen, USA

GeneRuler™ 100bp DNA Ladder

Fermentas International, Canada

Glycerol, 99.5% A.C.S. Reagent

Sigma Aldrich, Germany

LB Broth

Becton Dickinson, USA

Lipofectamine™ 2000 Reagent

Invitrogen, USA

Loading Buffer 5x

Elchrom Scientific AG, Switzerland

MgCl2 Solution 25mM

Roche, USA

Metafectene™ PRO

Biontex, Germany

Montage™ SEQ_{96} Sequencing Reaction Cleanup Kit

Millipore, USA

OPTI-MEM® I+GlutaMAX™-I1x

Gibco, UK

pBR322 DNA-*Msp* I Digestions
New England Biolabs, USA

PBS
Gibco, UK

PfuUltra™ Hotstart DNA Polymerase, *PfuUltra*™ HF Reaction Buffer 10x
Stratagene, USA

QIAprep® Spin Miniprep Kit, EndoFree® Plasmid Maxi Kit
Qiagen Inc., USA

Rapid-Load™ PCR Loading Dye
OriGene Technologies, USA

Restriction Enzymes HindIII (10U/µl), XbaI (10U/µl), XhoI (10U/µl)
Fermentas International, Canada

SYBR® Green I nucleic acid gel stain 10 000x
Molecular Probes, Netherlands

T4 DNA Ligase (5U/µl), 10x Ligation Buffer
Roche Diagnostics, Germany

5x TBE Buffer
Eppendorf, Germany

0.05% Trypsin-EDTA 1x
Gibco, UK

1.2. Cells and Cell Lines

HepG2, Hepatocellular Carcinoma Human

ATCC®, HB-8065
Lot: 4004528

One Shot®TOP 10
Invitrogen, Carlsbad CA, USA

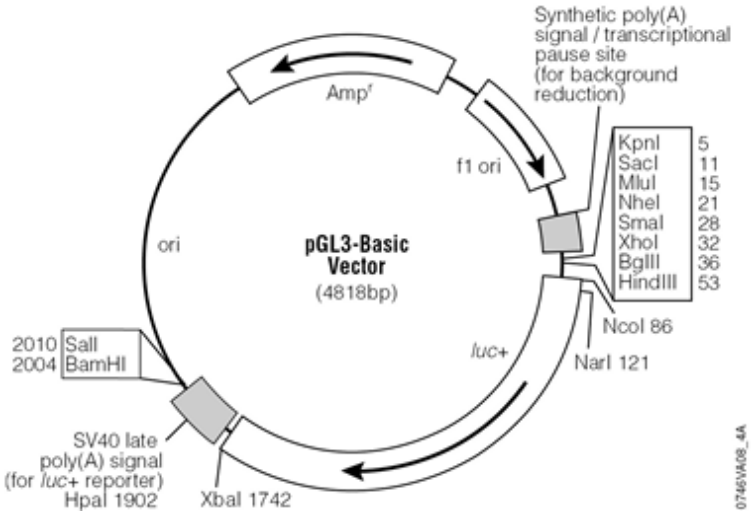
DH5α chemically competent cells
self-made by fellow student Harihara M.P.S.

1.3. Cloning Vectors and Constructs

pGL3 basic vector
Promega Corporation, Madison WI, USA

Description by the manufacturer:
The pGL3-basic vector lacks eukaryotic promoter and enhancer sequences, allowing maximum flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter upstream from *luc*+.

Base pairs	4818
Promoter	none
Enhancer	none
Multiple cloning region	1 to 58
Luciferase gene (*luc*+)	88-1740

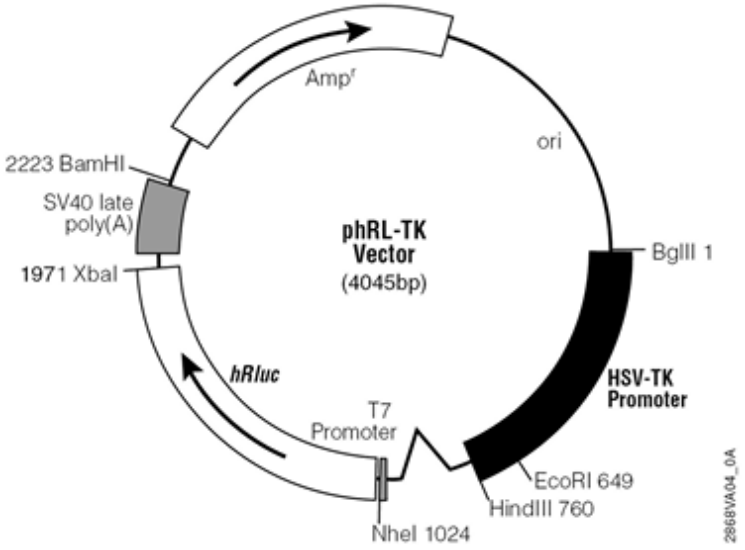


Two constructs with the pGL3-basic vector containing an inserted promoter had previously been made by a fellow student. They were used for the experiments in this thesis. These two constructs are **pGL3-A** and **pGL3-G**, both containing one of the two variants of the promoter SNP -1639G>A.

phRL-TK vector

Promega Coporation, Madison WI, USA

Base pairs	4045
HSV-TK promoter	7 to 759
Chimeric intron	826-962
T7 RNA polymerase promoter (-17 to +2)	1006-1024
T7 promoter transcription start site	1023
hRluc reporter gene	1034-1969



2. Methods

2.1. Preparation of Plasmids from Glycerol Stocks

The plasmids containing the wildtype (-1639G) and variant (-1639A) promoter were kept frozen at -80°C in a glycerol stock. They were thawed and propagated to be used in my thesis experiments.

A small aliquot of each construct was plated out on LB agar plates containing ampicillin (100µg/ml) directly from the glycerol stock. A few of the resulting colonies were picked and inoculated in 2ml liquid LB media each and then incubated at 37°C on a shaker for 16 hours.

The plasmid DNA was isolated following the protocol of the QIAPrep Spin Miniprep Kit using a microcentrifuge. According to the manufacturer, the Miniprep should yield up to 50µl or up to 20µg of plasmid DNA which is sufficient for sequencing and restriction enzyme digestion. To obtain enough material for transfection experiments, Endofree Plasmid Maxiprep preparation was performed.

For this procedure 100ml liquid cultures were inoculated also with colonies obtained from the glycerol stock.

2.1.1. Miniprep Procedure

Following lysis of bacterial cells, selective adsorption of the plasmid DNA onto a silica membrane and subsequent washing, the DNA was eluted from the column during a centrifugation step using an Eppendorf 5415 D microcentrifuge.

The starting materials for the Miniprep were 2ml liquid cultures inoculated with the colonies prepared from the glycerol stock. After 16 hours of incubation 1.5ml of these bacterial cultures were transferred to Eppendorf tubes, centrifuged and the cell pellets washed with three different buffers contained in the Qiagen Kit. The resulting supernatants were applied to QIAprep spin columns which were centrifuged and washed followed by elution of the plasmid DNA.

The eluted plasmid DNA was analyzed for right size and amount of DNA by gel electrophoresis on a 0.8% agarose gel. Electrophoresis was performed at 100V for 50min. A High Range DNA Marker was used to determine the size of the bands.

To make sure that not only the whole plasmid but also the inserted promoters were of the right length, they were cut out by restriction enzyme digestion with XhoI and HindIII in buffer R and ddH_2O. Restriction was performed by incubation at 37°C for 2 hours. Both restriction enzymes were used at a concentration of 10U/µl.

The resulting DNA fragments were also tested for right length on a 0.8% agarose gel.

2.1.2. Maxiprep Procedure

In the course of the Maxiprep plasmid DNA was isolated from 100ml liquid over night cultures using a Hettich Rotanta TRC centrifuge.

The cultures were transferred into 50ml Sarstedt tubes, centrifuged and the cell pellets washed three times. The resulting lysates were filtered and the plasmid DNA was thereby caught inside the filter membrane which was then washed to release all none DNA material. Plasmid DNA was eluted by application of elution buffer.

The plasmid and the insert cut out of the plasmid with XhoI and HindIII were analyzed on a 0.8% agarose gel. Running conditions for the gels were 100V for 50min.

2.1.3. Pooling of Plasmid DNA

The Maxi-Prep DNA samples were pooled and aliquoted into samples of 25 µl.

The OD of each pool was determined using a NanoDrop® ND-1000 spectrophotometer. The concentrations of the samples were later required for transfection of the HepG2 cells.

2.1.4. Preparation of new Glycerol Stocks

One ml of fresh LB media was inoculated with bacterial colonies that were picked for the plasmid preparations. Following 16 hours incubation the cultures were mixed with glycerol at a ratio of 60:30. For each of the constructs about 1ml of glycerol stock was prepared. The glycerol stocks were stored at -80°C.

2.2. Sequencing

2.2.1. Cycle Sequencing

The sequencing reaction was carried out using a Big Dye® Terminator v3.1 Cycle Sequencing Kit.

The sequencing PCR with four ddNTPs (ddATP, ddGTP, ddCTP, and ddTTP), was performed in a 96-well plate in a total volume of 10µl for each sample. This volume contained the Big Dye Sequencing Buffer and Ready Reaction Mix contained in the kit, 25ng of plasmid, 5pmol primer and deionized water. In every well only one primer was used. The following 6 primers were applied:

Designation	Position	Sequence 5'-3'
VKORC1 5'UTR3 fw	4225-4243	AGTGTAGATGGGGAGGATG
VKORC1 5'UTR4 rv	5025-5006	GGGAAATGAAGTCTCCACAG
VKORC1 5'UTR1 rv	4080-4961	CTTCCTCACTTCTTCCTTGC
VKORC1 5'UTRi fw	3514-3539	CCGCTCGAGTAGATGTGAGAAACAGCATCTGG
VKORC1 5'UTRi rv	5277-5260	CCCAAGCTTAAACCAGCCACGGAGCAG
VKORC1 5'UTR2 fw	3924-3938	CTGGGCGACAGAGT

Table 2. Primers used for Cycle Sequencing

The cycle sequencing reaction was performed on a PE 9700 Thermal Cycler. The temperature protocol was:
Denaturation for 1' at 96°C followed by 25 cycles of 10'' at 96°C, 5'' at 50°C and final elongation for 4' at 60°C. Finally the samples were cooled to 4°C.

2.2.2. Purification

Before sequence analysis the samples were purified using a vacuum pump. They were therefore transferred to a SEQ96 plate and diluted with 25µl of injection solution which was then sucked off at a pressure of 20bar. This procedure was repeated once.

2.2.3. Sequence Analysing

The instrument used for analyses of the sequences was an ABI PRISM® 3100 Genetic Analyser. The separation technique of these sequencing machines is capillary electrophoresis. The software program used to read the sequences was SeqScape®.

2.3. Cell Culture

The cell line used for the transfection experiments was a HepG2 cell line obtained from ATCC, which was derived from a 15 year old male Caucasian with a hepatocellular carcinoma.
The product was received in an ampule containing 1ml of 3.8 x 10^6 cells/ ml and treated according to the company recommendations. The cells

were thawed at 37°C for approximately 2 minutes. Under sterile conditions 500µl of the vial contents were transferred to a fresh 50ml tube containing 5ml of FBS and immediately centrifuged at 1200rpm for 5min. This procedure was repeated, the resulting cell pellet resuspended in 8ml of DMEM and transferred into a 75cm³ flask containing 8ml of DMEM plus geneticin. The culture was then incubated at 37°C. Media was changed twice a week.

2.3.1. Passaging

Depending on their confluence the cells were split (or passaged).
The culture medium was discarded and the cells washed once with 5ml of PBS. The cell layer was rinsed with 4ml of 0.25% trypsin/ 0.53mM EDTA solution. Then, 5ml of trypsin-EDTA was added and the cells were incubated for 15 minutes to disperse the adherent cell layer. Five ml of complete growth medium were added and the flask tilted a few times to release cells that still stuck to the bottom. The contents were transferred into a fresh 50ml Falcon tube and centrifuged at 1000rpm for 5min. The supernatant was discarded and the cell pellet resuspended in about 4ml of complete growth medium which was then distributed to two new 25cm^2 flasks.

2.4. Transfection of HepG2 Cells

2.4.1. Lipofectamine™ 2000 as Transfection Reagent

24 hours before transfection, the cultured cells were seeded into a 24-well plate with approximately 4 x 10^5 cells per well containing 500µl of growth medium without antibiotics each. The plate was incubated at 37°C.

For transfection 750ng of the pGL3 basic vector, the pGL3-A and the pGL3-G construct was used, of the phRL-TK vector 25ng were used. All samples were diluted in Eppendorf tubes with 50µl of Opti-MEM. After 5min incubation at room temperature Lipofectamine (diluted with Opti-MEM at a ratio of 1:25) was added to reach a total volume of 100µl. The samples were incubated for 20min at room temperature. They were then

added to the cells in the 24-well plate instead of the growth media. For each sample 4 transfections in 4 separate wells were conducted.
The plate was incubated for another 48hours at 37°C prior to the Luciferase Reporter Assay.

2.4.2. Metafectene™ Pro as Transfection Reagent

Metafectene™ Pro is a new reagent for efficiently transfecting moderately hard- or hard-to-transfect cell lines. It uses two new techniques that are called **TOP (Toxicity Optimization Module) -** and **RMA (Repulsive Membrane Acidolysis) -Technology**.

Transfection with Metafectene basically uses cationic lipids for intracellular gene transfer. These cationic lipids form a DNA-lipid-complex or lipoplex with the genetic material they are supposed to transport into the cell. The complex is then taken up by the target cell through endocytosis.
Afterwards the DNA resides in the cytoplasm in the form of lipoplex-containing endosomes. By osmotic disruption the lipoplexes are released into the cytosol. The plasmid DNA must now reach the nucleus but since it cannot penetrate the nuclear membrane by itself, transfection can only take place during cell division when the nuclear membrane opens.

With **RMA technology** the acidic environment caused by the breakdown of the endosomes is used to weaken the membrane structure of the lipoplexes. Repulsive forces between the positively charged lipophilic parts of the lipids then lead to the easy disruption of the membranes. Consequently a very high amount of naked genetic material is released into the cytosol.
TOP technology decreases the toxic effects of transfection throughout the whole process. This is very important to maximize the success of transfection. Furthermore it helps to destabilize the lipoplexes.

For transfection with Metafectene, the cells were seeded into a 24-well plate with 4 x 10^5 cells plus 500µl of medium without antibiotics per well.

For each well 1µl of Metafectene was diluted in 100µl of Opti-MEM. For the plasmid DNA samples 1µg of each construct, pGL3-basic, pGL3-A and pGL3-G, and 25ng of the phRL-TK vector were mixed with the Metafectene/ Opti-MEM solution and incubated at room temperature for 15min. Of the resulting solutions 100µl was transferred into each well of a 96-well microtiter plate. As with the Lipofectamine transfection, for every sample 4 separate wells were used. Four wells for phRL-TK and 4 wells for only Opti-MEM with Metafectene served as negative controls. The plate was incubated for 4 hours at 37°C. Afterwards 500µl of medium with 20% FBS was added to every sample. The plate was incubated for another 48 hours prior to the Luciferase Reporter Assay.

2.4.3. Preservation of Cells

Cells were preserved for later experiments. To do so, the media was removed and the cells were washed once with PBS. Then, Trypsin-EDTA was added to release the cells from the bottom of the flask. Medium containing FBS was added and the whole contents of the flask were centrifuged at 1200rpm for 5min. The supernatant was removed and the cell pellet was resuspended in DMEM containing 10% DMSO. The cells were stored at -80°C over night and transferred to liquid nitrogen the next day.

2.5. Dual-Luciferase® Reporter Assay

The Dual-Luciferase Reporter Assay was carried out to measure the expression level of VKORC1 in HepG2 cells with a wildtype promoter polymorphism (-1639G) and compare it to the expression level of the variant polymorphism (-1639A). The expression of two individual reporter enzymes is measured simultaneously. These enzymes are firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferase. Two liquid reagents are added to the sample that detect the luciferase activities and give a luminescent signal.

Prior to the assay the cells were lysed. The growth media was removed and 100µl of Passive Lysis Buffer were added to each transfected sample in the 24-well plate. The plate was put on a shaker for 15min. Afterwards 20µl of each sample were transferred into a 96-well plate which was then set in place in a luminometer. The two reagents necessary for the measurements, LAR II and Stop & Glo ® had been prepared before. In the process of measuring, first 100µl of LAR II are added to each well. This reagent detects the activity of the firefly luciferase and generates a luminescent signal. The subsequent addition of 100µl of Stop & Glo ® per well quenches the reaction of LAR II with the firefly luciferase and instead produces a luminescent signal from the *Renilla* luciferase.

2.6. Construction of a VKORC1 Full-Length cDNA

For the construction of the VKORC1 full-length cDNA the plasmids pGL3-G and pGL3-A which contained the VKORC1 promoter variants were used. The luciferase gene present in the pGL3 vectors was removed from the construct and was replaced by the VKORC1 cDNA.

2.6.1. RNA Isolation and Generation of cDNA

RNA was isolated from frozen HepG2 cells (see section 2.4.3., p.45) using the RNeasy® Mini Kit by Qiagen.

700µl of the frozen HepG2 cells were thawed in 2ml of Passive Lysis Buffer and 700µl of the resulting lysate were transferred into a QIAshredder spin column. To homogenize the lysate the column was centrifuged at 13200rpm for 2min. Afterwards the shredder was discarded and 1 volume of 70% ethanol was added to the flow-through. Then, 700µl of the sample were applied to an RNeasy spin column which was placed in a 2ml collection tube. The tube was centrifuged at 13200rpm for 30sec, the flow-through discarded, the column placed in a fresh collection tube and the tube centrifuged again at 13200rpm for 30sec. To remove contaminations the column was washed three times using wash buffers contained in the kit. The column was put into a fresh collection tube and centrifuged without addition of any reagent for 2min at 13200rpm.

RNA was eluted by 40µl of RNase-free ddH_2O to the column. Optical density of the sample was measured directly after isolation using a NanoDrop® ND-1000 spectrophotometer.

The cDNA synthesis was performed briefly as follows: starting material was 10ng of the RNA samples isolated before. These were incubated for 10min at 70°C on a thermo cycler and put on ice immediately afterwards. They were then filled up with ddH2O to reach a total reaction volume of 12µl.
A Mastermix Premix was prepared that contained 5x buffer at a concentration of 1x, dNTP Mix at a concentration of 1mM, 20pmol of Oligo dT Primers and DTT at a final concentration of 10mM. 23.7 Units of RNA Guard and 8Units of MMLV-RT were added. Of this Mastermix (MM) 12.75µl were added to the RNA and incubated for 10 min at 22°C, 45min at 37°C, 3min at 99°C followed by cooling on ice. Finally 37µl of ddH_2O were added to the sample, resulting in a total volume of around 60µl.

2.6.2. Control-Gene PCR

GAPDH or Glycerinaldehyd-3-phosphat-dehydrogenase served as a reference gene for the RNA and cDNA isolated from the HepG2 cells.
The two primers specific for the GAPDH gene were used for amplification at a concentration of 10pmol:

Designation	Sequence 5'-3'
GAPDH Gap-f	GAAGGTGAAGGTCGGAGTC
GAPDH Gap-r	GGGGATGGTGATGGGATTTC

The Mastermix included: 2µl 10x PCR buffer without $MgCl_2$ (25mM), 0.2µl 20mM/base dNTPs, 0.17µl AmpliTaq Gold Polymerase (5U/ µl), 0.8µl Rapid Load PCR Loading Dye and ddH2O in a total reaction volume of 20µl. Of the undiluted cDNA 2µl were added and the PCR was performed with the following temperature protocol:
Denaturation for 7' at 95°C followed by 38 cycles of 30'' at 95°C, 30'' at 60°C and 30'' at 72°C and final elongation for 10' at 72°C, cooling to 4°C.

Fragment length was analyzed by gel electrophoresis on a 5% polyacrylamide gel (Criterion Gel, Bio Rad) at 175V for 45min. A 1xTBE *Msp*I Marker was used.

2.6.3. Primer Design

Special primers, one forward and one reverse, were designed to include the restriction sites for the enzymes HindIII and XbaI into the sequence of the cDNA construct. The HindIII restriction sequence was positioned directly before the start codon and the XbaI sequence directly behind the stop codon of the coding sequence. The resulting primers were the following:

Designation	Sequence 5'-3'	Length
#182 VKORC1cdsfw	AAGCTTATGGGCAGCACCTGGG	21bp
#183 VKORC1cdsrv	TCTAGATCAGTGCCTCTTAGCCTTGCC	27bp

2.6.4. PCR Amplification of the cDNA

For PCR amplification of the cDNA with restriction sites 10pmol of the primers (#182, #183) and 100ng of the cDNA were used. The other reagents were: 2µl 10x PfuUltra PCR buffer, 0.2µl 20mM dNTP Mix, 0.5µl PfuUltra Hotstart Polymerase, Loading Buffer and ddH_2O to add to a total reaction volume of 20µl.

The temperature protocol was: Denaturation for 10' at 94°, 35 cycles of 30'' at 94°C, 30'' at 60°C and 2' at 72°C, final elongation for 7' at 72°C and cooling to 4°C. The amplified fragments were analyzed for right length on a 5% polyacrylamide gel at 175V for 45min. Again a 1xTBE *Msp*I Marker was used.

2.6.5. DNA Extraction from Agarose Gel

To minimize the risk of ligating a possibly wrong fragment into the vectors, the right PCR product was extracted from an agrose gel.

The gel slice was transferred into a small reaction tube, weighed, 3 volumes (in µl) of buffer QG were added to 1 volume (in mg) of gel. The mixture was then incubated for 10min at 50°C and vortexed every 2-3min. At the end of the incubation time the gel was completely dissolved. Following the addition of 1 volume of isopropanol the sample was transferred to a QIAquick spin column which was placed in a 2ml collection tube and was centrifuged for 1min at 13000rpm. The flow-through was discarded and the column put back into the tube. 0.5ml of buffer QG were added and the column was centrifuged again at 13000rpm for 1min to remove residual agarose. The DNA should by now be inside the column membrane. To wash the DNA 0.75ml of buffer PE were added and the tube was again centrifuged for 1min. The flow-through was discarded and the column centrifuged for an additional 1min to remove all traces of the flow-through. DNA was eluted by applying 30µl of buffer EB.

2.6.6. Restriction Enzyme Digestion of Plasmids and cDNA

The VKORC1 full-length PCR product, the plasmid pGL3-A and the plasmid pGL3-G were linearized by restriction enzyme digestion with HindIII and XbaI. Double digestion also ensures that the cDNA is later inserted in the right direction into the vector during ligation.

The digestion was carried out according to the protocol by Roche Applied Biosystems.

One Unit of each enzyme was mixed with 25µl of Buffer B. For every restriction 2.5µl of this mix was used. For digestion of the plasmids 1µg of pGL3-G and pGL-A was used, for digestion of the full-length PCR product 1.2µg were used. The reaction volumes were filled up with ddH_2O to 25µl. The samples were incubated on a thermo cycler at 37°C for 1 hour.

The digested plasmids were separated by agarose electrophoresis at 100V for 60min, and the bands corresponding to the correct fragment length were extracted with the QIAquick Gel Extraction Kit.

2.6.7. Ligation

The ligation of insert and vectors was performed with the Rapid DNA Ligation & Transformation Kit by Fermentas.

For the ligation 10µl reaction volumes were used that contained DNA ligase, ligation buffer, vector and insert. 70ng of pGL3-A and pGL3-G vector were mixed with 40ng of PCR product and 5U of T4 DNA Ligase (1µl). 4µl of 5x Rapid DNA Ligation buffer were added to reach a final reaction volume of 10µl. The ligation reaction was performed at 22°C in a thermo cycler for 5min. The products were analyzed on a 0.8% agarose gel at 100V for 90min. A High Range DNA Ladder was used to determine the lengths of the bands.
Transformation into bacterial cells was performed immediately afterwards.

2.6.8. Transformation into Bacterial Cells

For the transformation of the ligated plasmid constructs, chemically competent DH5α cells were used that were available in the lab. For the transformation a very short protocol was used. For each ligation product 50µl of the competent cells were thawed on ice. Once they had reached a homogeneous liquid consistency, 5µl of the ligation product was added. The reaction tubes were then put on a shaker that had been pre warmed to 37°C for 10min. After this incubation time the whole amount of the transformation products was plated out on pre warmed LB agar plates containing 100µg/ml of ampicillin. The plates were then incubated at 37°C for 12-16 hours.

2.6.8.1. Colony PCR

Selected colonies were tested by PCR to evaluate if the right insert had been ligated into the vectors. The PCR MM contained 10x GoldTaq Buffer, 25mM MgCl2, 5x PCR Loading Dye, 20mM dNTPs, 0.5U AmpliTaq Gold Polymerase, ddH2O, and 10pmol of each of the two primers that were specially designed to amplify the VKORC1 full-length construct. Colonies were picked and added to 20µl MM. PCR amplification was performed using the following temperature protocol: Denaturation for 10' at 94°C

followed by 35 cycles of 45'' at 94°C, 45'' at 60°C and 2' at 72°C and final elongation for 7' at 72°C. The samples were cooled down to 4°C. The products were tested on 5% polyacrylamide gels (Criterion, BioRad) at 175V for 45min.

Colonies containing the right plasmid were picked again and used to inoculate a 2ml LB medium aliquot which was incubated at 37°C for about 16 hours. Subsequently plasmid DNA isolation was carried out with the QIAPrep Spin Miniprep Kit by QIAGEN (for procedure see section 2.1.1., p. 35). Gel electrophoresis was performed on a 0.8% agarose gel at 100V for 60min.

2.7. VKORC1 Full-Length Construct

2.7.1. Restriction Enzyme Digestion

Three different restriction enzyme digestions were performed to test if the selected insert was taken up by the vector and was present in the plasmids. Three different restriction enzyme digests were set up; combined digestion with the restriction enzymes HindIII and XbaI, digestion with HindIII and digestion with XbaI only. For the double digestions an enzyme mix was prepared that contained 25µl of 10x Buffer B, 10 Units of Hind III and 10 Units of XbaI. Of this mix 2.5µl were added to 800ng of the plasmid and filled up with ddH_2O to a final volume of 25µl.
For the single digestions of each vector 800ng were mixed with 1Unit of HindIII or with 1Unit of XbaI. The final reaction volume of 25µl was obtained by the addition of ddH_2O.
Following incubation at 37°C for 1hour the fragment length was checked on a 0.8% agarose gel running at 100V for 60min. Again a High Range Marker was used.

2.7.2. PCR

To test whether the promoter sequence and the VKORC1 coding sequence are present in the plasmid three different PCR mastermixes with

different primers were prepared. The first MM contained the VKORC1 c-fw primer for the promoter and the rev primer complimentary to a sequence in the VKORC1 cDNA. If the VKOR cDNA was successfully inserted adjacent to the promoter these two primers should produce a fragment of around 2200bp.
The second MM contained forward and reverse primers for the promoter element. This reaction served as a positive control.
The third MM contained forward and reverse primers for the VKORC1 coding sequence. This reaction also served as a positive control.

The PCRs were all performed in a reaction volume of 20µl:

1. 12.4µl ddH_2O, 2µl 10x GoldTaq Buffer, 1.5µl 25mM $MgCl_2$, 0.8µl 5x Rapid Loading Buffer, 0.2µl dNTP Mix, 1µl #183 VKORC1cdsrv (10pmol/ µl), 0.4µl #85 VKOR -1639 c-fw (10pmol/ µl), 0.1µl GoldTaq Polymerase (5U/ µl) and 1µl plasmid (~12.5ng).

Primer	Sequence 5'-3'
#183 VKORC1cdsrv	TCTAGATCAGTGCCTCTTAGCCTTGCC
#85 VKOR -1639 c-fw	ACAGTAAGGGATCCCTCTGGGAAGTC

PCR temperature protocol:
10' at 94°C, 35 cycles of 45'' at 94°C, 45'' at 60°C and 2' at 72°C, 7' at 72°C.

2. 12.4 µl ddH_2O, 2µl 10x GoldTaq Buffer, 1.5µl 25mM $MgCl_2$, 0.8µl 5x Rapid Loading Buffer, 0.2µl dNTP Mix, 0.5µl #83 VKOR -1639 w-rv (10pmol/ µl), 0.8µl #84 VKOR -1639 mut-rev (10pmol/ µl), 0.4µl #85 VKOR -1639 c-fw (10pmol/ µl), 0.1µl GoldTaq Polymerase (5U/ µl) and 1µl plasmid (~12.5ng).

Primer	Sequence 5'-3'
#85 VKOR -1639 c-fw	ACAGTAAGGGATCCCTCTGGGAAGTC
#84 VKOR -1639 mut-rv	AGGATTATTAGCGTGAGCCACCGCTCCT
#83 VKOR -1639 w-rv	AGGCGTGAGCCACCGCAACC

PCR temperature protocol:
10' at 94°C, 35 cycles of 45'' at 94°C, 45'' at 60°C and 2' at 72°C, 7' at 72°C.

3. 12.4µl ddH_2O, 2µl 10x GoldTaq Buffer, 1.5µl 25mM $MgCl_2$, 0.8µl 5x Rapid Loading Buffer, 0.2µl dNTP Mix, 1µl #182 VKORC1cdsfw (10pmol/ l), 1µl #183 VKORC1cdsrv (10pmol/ µl), 0.1µl GoldTaq Polymerase (5U/ µl) and 1µl plasmid (~12.5ng).

Primer	Sequence 5'-3'
#182 VKORC1cdsfw	AAGCTTATGGGCAGCACCTGGG
#183 VKORC1cdsrv	TCTAGATCAGTGCCTCTTAGCCTTGCC

PCR temperature protocol:
10' at 94°C, 35 cycles of 45'' at 94°C, 45'' at 60°C and 2' at 72°C, 7' at 72°C.

All PCR products were subsequently analyzed on a 5% Criterion gel at 175V for 45min using a 1xTBE *Msp*I Marker.

2.7.3. Sequencing

To identify the sequence of the two plasmids cycle sequencing was performed using the following primers:

Primer	Sequence 5'-3'
#182 VKORC1cdsfw	AAGCTTATGGGCAGCACCTGGG
#183 VKORC1cdsrev	TCTAGATCAGTGCCTCTTAGCCTTGCC
VKORC1 5'UTR 5fw	TCGCTGTTTTCCTAACTCG

Primers were used at a concentration of 5pmol. The plasmid DNA concentration was 25ng.

The reactions were prepared in a 96-well plate with 2µl Big Dye Sequencing Buffer (5x), 0.4µl Big Dye Terminator Mix and deionized water. The total volume in each well was 10µl.

The sequencing PCR was performed in a PE 9700 Thermal Cycler with the following temperature protocol: Denaturation for 1' at 96°C, 25 cycles of 10'' at 96°C, 5'' at 50°C, 4' at 60°C and cooling to 4°C.

The sequences were analysed on an ABI PRISM® 3100 Genetic Analyser.

V. RESULTS

1. Plasmid Recovery

1.1. Miniprep

When the frozen plasmid Glycerol stocks were reactivated and the isolated plasmids were analyzed by agarose gel electrophoresis after Miniprep and restriction enzyme digestion, it was observed that some plasmid molecules migrated with a longer fragment length than expected. The expected size of the inserted promoter region should be about 1700kb but was actually near 4000kb compared to the High Range DNA marker. This phenomenon was observed for the wild type as well as variant promoter construct.

We suspected that in some vectors the promoter segment had been inserted more than once in tandem fashion. For further experiments, only plasmids were chosen that migrated with the correct length.

With the new plasmids fresh glycerol stocks were prepared.

1.2. Sequencing

Clear sequences were obtained with low background noise. Expectedly, the two plasmids differed only by the one base that constitutes the polymorphism -1639G>A. The wild type sequence contained a G at the position -1639, the variant showed the base A in the same place.

2. Dual Luciferase® Reporter Assay

For the Dual Luciferase Reporter Assay HepG2 cells were transfected with plasmids pGL3-G and pGL3-A 48 hours prior to the performance of the assay. According to our results, the effect of the promoter variant on the expression of luciferase activity in HepG2 cells seemed to depend on the transfection method used. Following transfection with lipofectamine the promoter variant pGL3-A induced a higher luciferase activity while with Metafectene the pGL3-G promoter sequence induced the higher luciferase activity. The reason for these discrepant finding is currently unclear.

2.1. Lipofectamine™ 2000

Using lipofectamine as transfection reagent the luminescence signals were low and differed considerably between samples even though all samples were transfected under the same conditions.

No treatment	**PGL3 basic**	**pGL3-G**	**pGL3-A**
0.2	3	0	13
-1	1	-10.5	138
3.333	4	49	-11
1.333	-1	-17.75	0
		5.188	**35**
0	0	-39.5	-37.5
1.5	-1	6	42
0	0	27.929	70
0.25	-3.5	27	26.5
		5.357	**25.25**
		5.273	**30.125**

Table 3. Relative Luminescence Values following Transfection with Lipofectamine

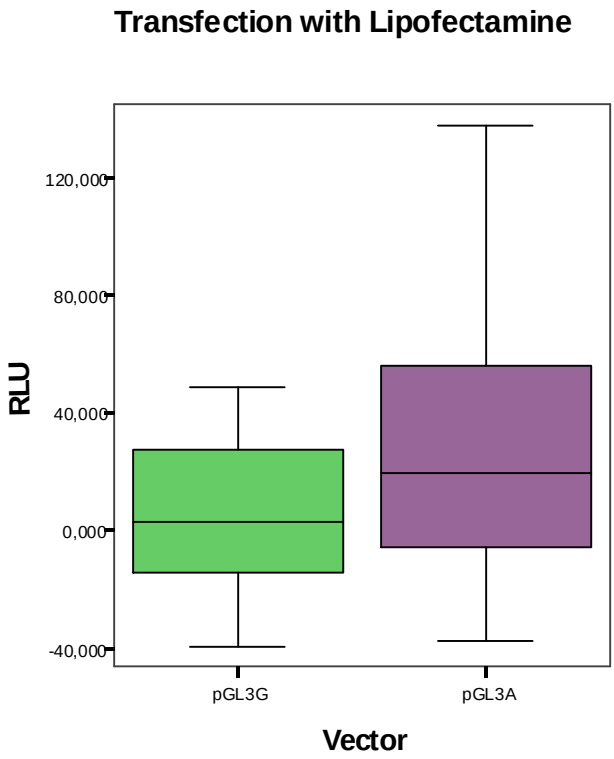


Fig.5. Relative Luminescence of cells transfected with Lipofectamine

Using lipofection, the pGL3-A promoter construct induced an 83% higher luciferase activity compared to the pGL3-G construct in HepG2 cells.
However, the transfection efficiency was very low and the data were highly variable. The results indicate that transfection of HepG2 cells using Lipofectamine does not give reliable results. Apparently, transfection is not always successful and cells do not always take up the construct. For our plasmid and HepG2 cells lipofection was not suitable.

2.2. Metafectene™ PRO

The transfection was repeated with the new transfection reagent Metafectene PRO which is described by the manufacturer as reagent suitable for hard-to-transfect cells.
The luminescence signals obtained with Metafectene were much higher than those obtained using lipofection but still differed between samples. Transfection efficiency was more consistent but still variable.

No treatment	PGL3 basic	pGL3-G	pGL3-A
0.004	1.119	400.078	361.352
-0.002	4.25	194.648	149.893
0.005	1.334	163.981	409.693
		252.902	**306.979**
-0.004	2.267	641.107	282.195
-0.001	2.257	73.679	312.552
-0.002	0.681	333.702	102.233
-0.002	0.925	283.996	616.266
		333.121	**328.312**
0.002	0.157	70.252	63.808
0	0.518	43.897	27.565
-0.004	0.158	15.665	35.4
0.003	0.498	206.036	55.155
		83.963	**45.482**
-0.001	0.65	63.396	92.195
0.002	0.276	112.542	34.103
0.001	0.997	59.184	72.212
0.01	0.272	254.473	55.685
		122.398	**63.548**
		198.096	**186.080**

Table 4. Relative Luminescence Values for the Transfection with Metafectene

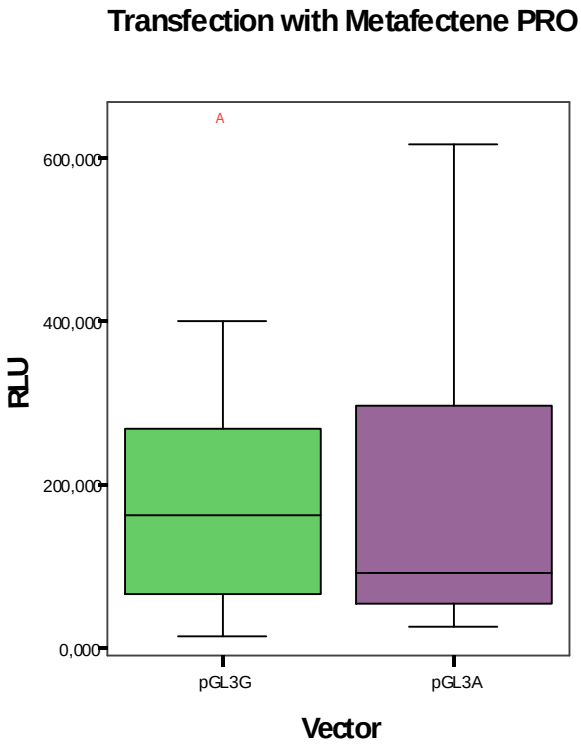
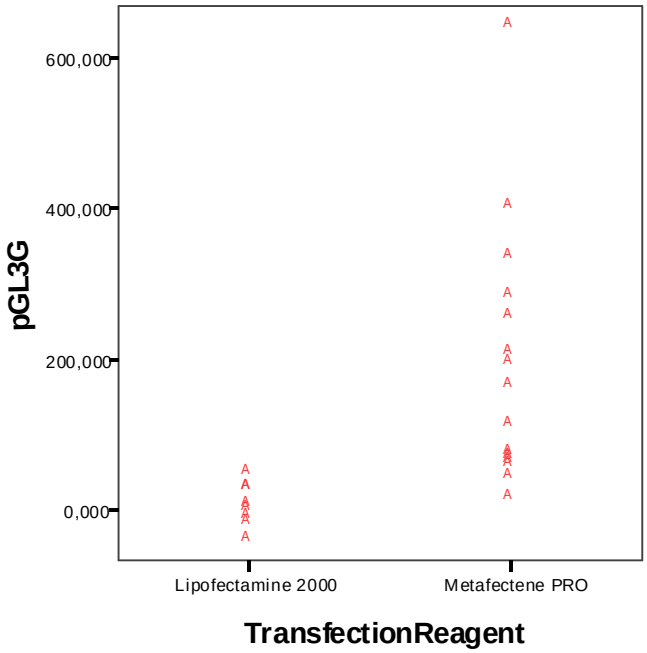


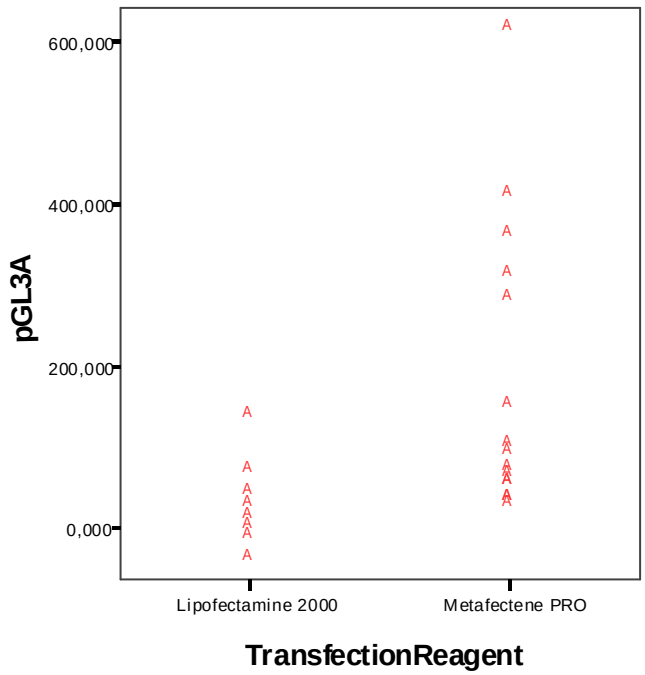
Fig.6. Relative Luminescence by cells transfected with Metafectene

With Metafectene as transfection reagent the pGL3-G promoter construct induced only a 6% higher luciferase activity compared to the pGL3-A construct. This is not a significant difference.

According to my results the variations at -1639 of the VKORC1 promoter does not seem to have a significant effect on the expression of luciferase activity in HepG2 cells.

2.3. Comparison of the Transfection Reagents





The comparison of the two transfection methods shows that transfection of HepG2 cells with Metafectene is more efficient for pGL3-A and pGL3-G than using lipofection. Nevertheless, the quadruplicate tests indicate a high variability of the performance of each Metafectene transfection. Possibly, modifications of the protocol could improve the transfection efficiency.

3. Generating a VKORC1 Full-Length Construct

3.1. VKORC1 Coding Region cDNA with Restriction Sites

Fig.9. shows the PCR products obtained by amplification of the cDNA with the primers containing the restriction sequences of HindIII and XbaI.

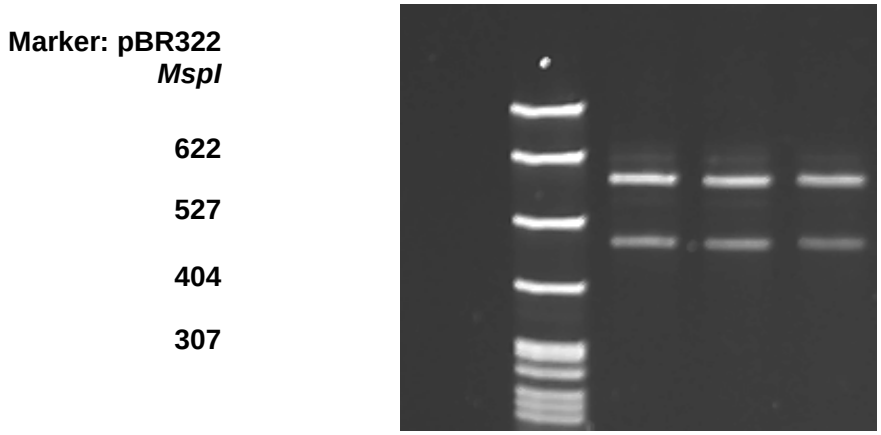


Fig.9. Fragments generated by PCR amplification of cDNA with restriction sites

I observed that two PCR fragments were generated. The upper band represented the expected fragment which has a length of 504bp, made up of 492bp for the VKORC1 coding region and 6bp for each of the two restriction sites. The shorter band shows a fragment length of about 380bp.

A partially complementary sequence to the forward primer was identified about 100bp within the coding sequence of VKORC1. Even though only a

few bases corresponded with the selected primer, this was sufficient to generate a second binding site.

The use of different annealing temperatures did not change the results. Other modifications such as a surplus of the reverse primer also did not prevent the generation of the second fragment. Thus, the correct fragment was excised and extracted from the agarose gel prior to further use.

3.2. Linearising of the Plasmid Vectors and Removal of the Luciferase Gene

Linearising of the plasmid vectors and removal of the luciferase gene was accomplished by restriction enzyme digestion with HindIII and XbaI and as confirmed by gel electrophoresis. The gel picture showed fragments with a length of around 4818bp which corresponds to the right size.

3.3. Ligation of the VKORC1 Coding Region into Plasmid Vectors

The success of the ligation was examined by gel electrophoresis following digestion with HindIII and XbaI.

Fig.10. shows undigested and digested plasmids. In slots number 3 and 8 the bands for pGL3-G and pGL3-A digested with both HindIII and XbaI can be seen. The upper band at around 4800bp represents the linearised vectors. No band is visible for the insert, yet there is a second band at about 1800bp.

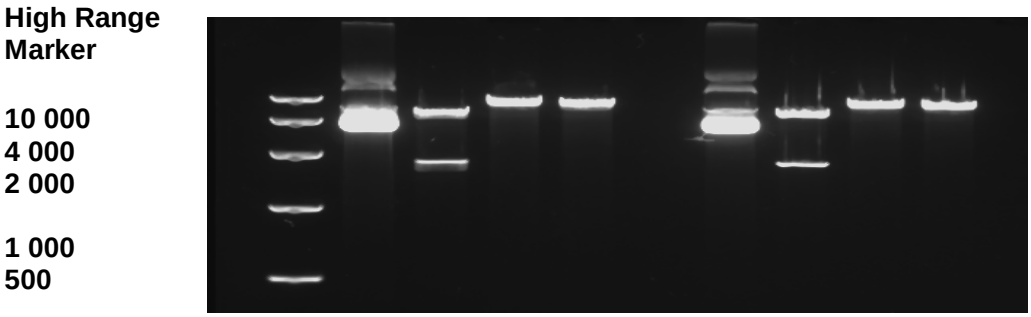


Fig.10. Slot1: Marker, Slot2: pGL3-G undigested, Slot3: pGL3-G double digested, Slot4: pGL3-G digested with HindIII, Slot5: pGL3-G digested with XbaI, Slot7: pGL3-A undigested, Slot8: pGL3-A double digested, Slot9: pGL3-A digested with HindIII, Slot10: pGL3-A digested with XbaI

4. Revision of VKORC1 Full-Length Construct

4.1. PCR Amplification and Gel Electrophoresis

Fig.11., 12. and 13. show the results of the three PCR reactions performed to check if the insert was present in the constructs and if it was integrated adjacent to the promoter instead of the luciferase gene.

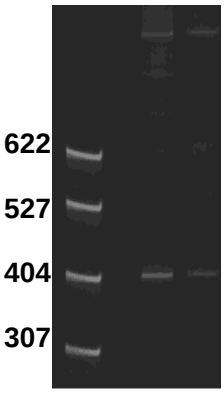


Fig.11. PCR results for promoter fw primer and insert rev primer

The gel in Fig.11 shows two bands, one at about 400-500bp and one in the high molecular weight range – top of the gel, difficult to see. The photo shows a polyacrylamide gel for which the standard marker is the *MspI* marker with the highest band at 622bp. A correct estimation of the length of this fragment was not possible because the product was out of the range of the markers used. The expected length of the PCR product should be about 2200bp. While the upper band could represent the right fragment the source of the smaller band was unclear – such a band should not be there.

To determine the length of the high molecular weight product the electrophoresis was repeated on an agarose gel using a High Range marker but this time, only the smaller product was visible.

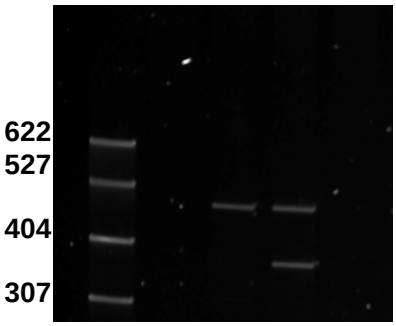


Fig.12. PCR results with insert primers

In Fig.12 in slot number four two bands can be seen. The upper band at around 500bp represents the insert, the shorter fragment at about 380bp is the product generated by the binding site of the fw primer within the sequence.

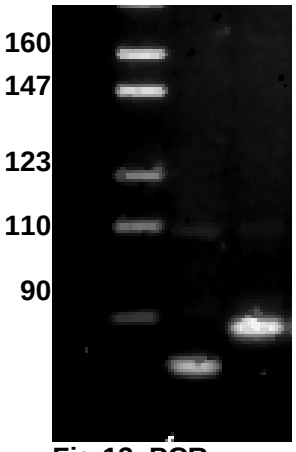


Fig.13. PCR results with promoter primers

Fig.13 shows the PCR products for the PCR with only the promoter primers. In slot number two the band for the wild type promoter pGL3-G with a length of 82bp can be seen, in slot three the band for the promoter of the construct pGL3-A with a length of 88bp is visible.

4.2. Sequencing

Sequence analysis of the constructs indicated incorrect sequences. A DNA fragment corresponding to part of the luciferase gene was identified between the VKORC1 promoter and the VKORC1 cDNA.

Apparently, the restriction enzyme digestion did not remove all of the luciferase gene sequence. A part of the sequence of the luciferase gene remained in the construct. To my surprise, the luciferase gene sequence was followed by a sequence that does not correspond with the VKORC1 promoter nor the VKORC1 cDNA nor the luciferase gene.
To determine to which gene this sequence was corresponding I did a blast search using the internet platform NCBI. No similarity to any sequences in this database could be found neither in forward nor reverse orientation.

VI. DISCUSSION

The enzyme VKORC1 is the rate-limiting factor of the vitamin K cycle. Its availability is vital for the carboxylation of the VKD proteins which gurantee the proper function of the blood coagulation system. If the carboxylation of the VKD proteins is disturbed because of deficiencies in the expression of VKORC1, cardiovascular diseases, thromboembolic events and even osteoporosis can develop as a result. Some of these diseases can be treated or prevented by medication with anticoagulants such as warfarin.

In the course of this diploma thesis I tried to conduct similar experiments as Yuan *et al*.[52] to see if I was able to reproduce and maybe re-evaluate their results. I also used a HepG2 cell line, carried out transfection with Lipofectamine™ 2000 and analysed the constructs in a Reporter Gene Assay.
The reagent Lipofectamine proved unsuitable for this reaction as transfection efficiency was very low and highly variable. The values differed considerably from one assay to the next. The average expression promoted by the A allele was approximately 83% higher than by the G allele. If the results were reliable, this would be a contradiction to the results reported by Yuan *et al*. However, as the luciferase activity levels were so variable, I decided not to trust the values and instead chose to try another transfection reagent.

Metafectene™ PRO, a new and improved reagent appeared to be a very reliable product as it had higher transfection efficiency and therefore the values I obtained from the luciferase assay also were much higher. Although the results were still quite variable, I was able to use them for calculating the difference in expression levels between the SNP -1639G and -1639A. I found that the expression of the cells transfected with the -1639G promoter construct was only 6% higher than of those with the variant -1639A. This difference is not statistically significant which means that according to my experiments the SNP G-1639A does not have a significant influence on the promoter activity and expression of the

VKORC1 gene in HepG2 cells. These results disagree with those of Yuan *et al*. but agree with the findings of Bodin *et al*.

One of my fellow students at the KIMCL worked on a project very similar to mine. She conducted the same experiments but used osteoblasts instead of HepG2 cells and also experienced difficulties transfecting her cell line with Lipofectamine as transfection reagent. With Metafectene she was able to show a higher promoter activity of the VKORC1 G construct compared to the A variant.

Comparing these results to my own I draw two conclusions:
Firstly, Lipofectamine™ 2000 seems to be an unsuitable transfection reagent for HepG2 cells and I would recommend that scientist preferably use other methods for transfection of these cells.
Secondly, I suspect that the effect of the promoter polymorphism G-1639A is tissue-specific since it was observed that it does have an influence on the expression of the VKORC1 gene in osteoblasts but not in HepG2 cells.

The experiments I conducted in the first part of this diploma thesis focused on VKORC1 constructs that contained only the promoter of the gene with the SNP. In these constructs the promoter drove the expression of the luciferase gene and the resulting expression was measured in the Dual Luciferase Reporter Assay.
To find out if the results are different if the VKORC1 gene coding sequence is present in the construct and is regulated by the promoter we decided to prepare a construct containing the promoter and the VKORC1 cDNA. We chose to remove the luciferase from our plasmid DNA and replace it by coding region of the VKORC1 gene.
Producing the full length constructs proved to be a very complicated process. Several modifications were necessary to obtain the full length cDNA and ligate it with the promoter contained in the plasmid. Eventually, constructs were available and could be examined. Interestingly, I discovered that only parts of the luciferase gene had been removed by the restriction enzymes but some luciferase fragments were still present in the

construct. Surprisingly, the VKORC1 coding region sequence was not detectable adjacent to the promoter nor adjacent to the luciferase sequence. Even more astounding was the fact that the sequence corresponding to the luciferase was followed by an unidentifiable sequence that does not resemble any human gene sequence.
Thus, it was amazing that the VKORC1 cDNA could be amplified from the plasmid DNA by PCR. Possibly, it was built into the vector in the wrong place. Another explanation could be that the sequence of 500bp is too short and makes the vector unstable. Up to date it remains unclear to which gene or part of a gene the unidentifiable sequence corresponds. For time reasons, I was not able to test whether this sequence was already present in the luciferase vector. Such a control experiment should clarify whether the observed sequence was coming from the vector and whether the VKORC1 cDNA was integrated behind the segment luciferase/ unknown sequence instead of adjacent to the promoter. For the time being I want to conclude from my work that another strategy for the production of a promoter – cDNA construct of the VKOR gene has to be used.

In 2008 Wang *et al.*[48] prepared a construct. They used the complete VKORC1 gene to generate a transfectable construct and they succeeded. Presumably, the whole gene has to be used and the coding region alone is just not enough.

In conclusion, I believe that the results one can obtain from a reporter gene assay for the VKORC1 promoter function, eg using a Luciferase assay, are only partly meaningful because the promoter seems to be tissue dependent and this may not be completely reflected by the Luciferase assay. To study the regulation and function of VKORC1 in different cell populations one should produce full length VKORC1 constructs composed of promoter, exons and introns of the gene. To obtain such constructs one could follow the protocol reported by Wang *et al.*[48]

VII. APPENDIX

1. VKORC1 Full Sequence

VKORC1 Color FASTA, available at

http://pga.gs.washington.edu/data/vkorc1/vkorc1.ColorFasta.html

```
CAGAGGACAA CATAGATAGG TTCGGAGACT ACTCAGAGAA AGGGAGAGAG 50
AATGCTAGGT TATCTTTCAG CTGTTTTAGC TGCTTATTGT TCCAGCTTTA 100 | REPEAT
GCCACCATCT GACTGCAATT GTATGAGCCA GAAATAGCCA GGTAAAATTG 150
TCTTCCCAAA TTCCTAGTGC ACAAAAACTG TCAGAGGCTG GGGGCAGTGG 200 | REPEAT
CTCACTCCTG TAATCCCAGC AGCACTTTGG GAAGCCAGGG TGGGAGAACT 250
GCTTGAGCCC AGGAGTTAGA GGCAACATAA CAACAACTCA TCTCTAATGA 300 | var(298):[C:0.01]
AAAAAAAAAA AAAAGAAAGA AAAAGGCCAG GCACAGTGGC TCACGCCTGT 350 | REPEAT
AATCCCAGCA CTTTAGGAAG CCAAGGAGGG TGGATCATGA GGTCAGGAGT 400 | var(381):[C:0.47]
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AAAAATCAGC CACGCATAGT GGCTGGTGCC TGTAATCACA GCTACTCAGG 500
AGGCTAAGGC AGGAGAATCA CTTGAATCCG GGAGGCAGAG GTTGCAGTGA 550
GCCGAGATTG CACCACTGCA TTTCAGCCTG GGCAGCAGAG TGGGACTGTC 600
TCACATTAAT TAATTAATTG ATTAAATGCT AGAAGAAAAT ATGGTCAAAA 650
TCTTTTATAA CTCTTGGAGT GAGGAAGGCA ATCTCGGCTC ACTACAACTT 700 | REPEAT
CCACCTCCCG GGTTCAAATG ATTCTCCTGC CTCAGCCTCC TGAGTAGCTG 750 | var(740):[T:0.01]
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GAGACGAGGT TTCGCCATGT TGGCCAAGCT TGTCTTAAAC TCCTGACCTC 850
AGGTGATCCA CCCACCTCAG CCTCCCAAAT TGCTGGGATT ACAGGCATGA 900 | var(861):[A:0.26]
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CCACGTTGTC CAGACCTGTC TCAAACTCCT GGGCTCAAGT CATTGTCTTG 1000 | REPEAT
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CTAATTTTGT TTATTTTTTA TAGAGTGAGG GTCTCCCTGT GTTGTCCAGG 1100
GTGTCTCAAA CTCCTGGGCT CAAGTGGTCC TCCTGCCTCA GCCTCCCAAA 1150
GTGCTGGGAT TATAGGCATA AGCCACTGCA CCTGGCCCCA GTGTGTGTCT 1200 | REPEAT
TCTGAGGCTA AGTCATAAAG AGATTGCGGC TTCTATTTTG CTTCCTCTTA 1250
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AGATCAGAGA AAGCTGAGAT ACAGGCCTAA TGCCCAGTCC TCTGTATTCA 1350 | REPEAT
TTTCTAATTG CTGCTGTAAC AAGTTACTGC ACATTTAATG ATGTAAAACA 1400
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CTGGGCTGAA ATCAAGATGT TGATAGTTCC TTCCCGGTGA CTCTAGAGTG 1500
AGAATCCAGT TCTTTACCCT TTCTTGCCTT TAGAGGCCAC CCGTATTTAT 1550
TAGCTCACAA TCCCCTTCCT CCATCTTCAA ACCAGAAACA TTGCAGCTCT 1600
CTGTGTCTTT TTTCTTTACT CACATCTCCC TCTGACTTTC TTCTGCCATC 1650
CTCCTTCATT TTTTAAGGAC CCCTGTGGGC CAGGCATGGT GGCTTATGCC 1700 | REPEAT
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GGAGTTCCAG ACCAGCCTGG CCAACATGGC AAAACCCCAT CTCCACTGAA 1800
AATACAAAAA TTGGGCCAGG CACGGCGGCT CACACCCGTA ATCCCCATAC 1850 | REPEAT
TTTGAGAGGC TGAGGCAGGT GGATCACTTG AGGTCAGGAG TTCAAGACCA 1900
GCCTGGCCAA CATGGTGAAA TCCCGTCTCT ACTAAAAAAA AAAAAAATTA 1950 | NOT SCANNED
CAATAATTAG TCAGGCGTGG TGGCCAGCAC CTATAATCCC AGCTACTTGG 2000
```

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AGCTGAGATT GCACCACCGT ACTCCAGCCT GGATGACAGA GCAAGACTCT 2100
GTCTCAAAAT AAAAATTAAG ATAAATAATA AAAAATAAAT AAAAATCAGC 2150 | REPEAT
AAATTAGGCC AGGTGTGGTG GTGGCTCATG CCTGTACTCC CAGCACTTTG 2200 | REPEAT
AGGTGGGAAG GTTGCTTGAA ACCAGAAGTT TAGACCAGCC TGGGAAACAA 2250
AGTGAGACCC CATCTCTACA AAAATAAAAA TAAATTAGCC AGGTGTGGTG 2300
GGACGTACTT GTACTCCTAG CTACTAGGGA GGCTGAGGTG CAAGAATCGC 2350
TTGAACCTGG GAGACGGAGG TTGCAGTGAG TTATGACAGT GCCACTACAC 2400
TCCAGCCTGG GTGTCAAGGG TCTTTGAAAA AATAAACAAA ATAGGCTGGG 2450 | REPEAT
*CGTGGTGGCT TACACTTGTA ATCCC*AGCAC TTTGGGAGGC TGAGGCAGGC 2500
AGATCACAAA GTCAGGAGTT CGAGACCAGC CTGGCCAACA CAGTGAAACC 2550
CCGTCTCTAC TAAAAATACA AAAATTAGCC AGGCATGGTT GCACGCGCCT 2600
GTAGTCCCAA CTGCTCGGGA GGCTGAGGCA GGAGAATGGC TTGAACCTGG 2650
GG**G**GGGGCGG AAGTTATAAT GAGCCAAGAT TGTGCCACTG CACTCCAGCC 2700 | **var(2653):[C:0.17]**
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CCCAGGTGGG TGGATCATCT GAGGTCAGGA GTTTGAGACC AGCCTGACCA 2850
ACATGGAGAA ACCCCATCTC TACTAAAAAT ACAAAAAATT AGCCAGGCGT 2900
GGTGGCACAT GCCTATAATC CCAGCTACTC AGGAGCCTGA GGCAGGAGAA 2950
TCGCTTTAAC CGGGGAGGTG GAGGTTGCGG TGAGTCGAGA TAGCACCATT 3000
GCATTCTAGC CTGGGCAACA AGAGCAAAAC TCCATCTCAA AAATAAATAA 3050
ATAAATAGAT AGATAGGCTG GGCACGGTGG CTCACGCCTG TAATCCCAGC 3100 | REPEAT
ACTTTGGGAG GCCGAGGTGG GCGGATCACC TGAGGTCAGG AGTTCAAGAC 3150
CAGCCTGGCC AATATAGCGA AACCCTGTCT CTACTAAAAA TTTAAAAAAT 3200
TAGCCAGGCA TGATGGCGGG TGCCTGTAAT CCCAGCTACT CGGGAGGCTG 3250
AGGCAGGAGA ATCTCTCGAA CCTGGCAGGC GGAGGTTGCA GTGAGCCGAG 3300
ATCACACCAT TGCACTCCAG TTGGGCAACA AGAGCGAAAC TCCACCTCAA 3350
AAACAAAATA AAATAAAATA AAATCAGGAG ATTGGTCAGC TTAATTCCAT 3400 | REPEAT
CTGCAACCTT AATTCCCTTT TGGCCATTTA ACCT**A**TCATA TTCACAAGTT 3450 | **var(3435):[G:0.14]**
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AGGGTAGGTG CAACAGTAAG GGATCCCTCT GGGAAGTCAA GCAAGAGAAG 3650 3673G>A-com-fw
ACCTGAAAAA CAACCATTGG CC**G**GGTGCGG TGGCTCACGC CTATAATCCT 3700 | REPEAT |
var(3673):[A:0.29] 3673G>A-wt-rev 3673G>A-mut-rev
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GACAAGCCTG GCCAACATGG TGAAACCCTG TCTCTACTAA AAATACAAAA 3800
ATTAGCCAGA CATGGTGGCA GGCACCTGTA GTCCAAACTA CTTGGGAGGC 3850
TGAGGCACGA GAACCACTTG AACCCTGGAG GCGGAAGTTG CAGTGAGCCG 3900
AGATGGCACC GCTGCACTCC AGCCTGGGCG ACAGAGTGAG ACTCCGTCTC 3950
AAAATAAGTA AATAAATAAA TAAATAACCA TTGGGTCTAG CGTCTTGGTG 4000 | REPEAT
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GGATACTGTC AAGGGTGTGT GTGGCCAAAG GAGTGGTTCT GTGAATGTAT 4450
GGGAGAAAGG GAGACCGACC ACCAGGAAGC ACTGGTGAGG CAGGACCCGG 4500

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ATGCTTGGTT CCCGAATCGG ATCGCCGTAT TCGCTGGATC CCCTGATCCG 4600 5'UTR-fw
CTGGTCTCTA GGTCCCGGAT GCTGCAATTC TTACAACAGG ACTTGGCATA 4650
GGGTAAGCGC AAATGCTGTT AACCACACTA ACACACTTTT TTTTTTCTTT 4700 | REPEAT
TTTTTTTTTG AGACAGAG**T**C TCACTCTGTC GGCCTGGCTG GAGTGCAGTG 4750 | **var(4719):[C:0.02]**
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CCTGCCTCAG CCTCCCGAGT AGCTGGGATT ACAG**G**CATGT GCCA**C**CACGC 4850 | **var(4835):[A:0.01**] | **var(4845):[A:0.01]**
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CAAAGTGCTG GGATTACAAG CGTGAGCCAC CGTGCCCGGC CAACAGTTTT 5000
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ACAACTCCCA TCATGCCTGG CAGCCGCTGG GGCCG*CGATT CCGCACGTCC* 5100 | Exon 1 | *UTR*
CTTACCCGCT TCACTAGTCC CGGC*A*TTCTT *CGCTGTTTTC CTAACTCGCC* 5150
CGCTTGACTA GCGCCCTGGA ACAGCCATTT GGGTCGTGGA GTGCGAGCAC 5200
GGCCGGCCAA TCGCCGAGTC AGAGGGCCAG GAGGGGCGCG GCCATTCGCC 5250
GCCCGGCCCC TGCTCCGTGG CTGGTTTTCT CCGCGGGCGC CTCGGGCGGA 5300 E1-fw
*ACCTGGAGAT A*ATGGGCAGC ACCTGGGGGA GCCCTGGCTG GGTGCGGCTC 5350
M G S T W G S P G W V R L 13
GCTCTTTGCC TGACGGGCTT AGTGCTCTCG CTCTACGCGC TGCACGTGAA 5400 5'UTR-rev
A L C L T G L V L S L Y A L H V K 30
GGCGGCGCGC GCCCGGGACC GGGATTACCG CGCGCTCTGC GACGTGGGCA 5450
A A R A R D R D Y R A L C D V G 46
CCGCCATCAG CTGTTCGCGC GTCTTCTCCT CCAGGTGTGC ACGGGAGTGG 5500 LC-VKORC1-fw
T A I S C S R V F S S R 58
GAGGCGTGGG GCCTCGGAGC AGGGCGGCCA GGATGCCAGA TGATTATTCT 5550 E1-rev
GGAGTCTGGG ATCGGTGTGC CCGGGGAACG GACACGGGGC TGGACTGCTC 5600
GCGGGGTCGT TGCACAGGGG CTGAGCTACC CAGCGATACT GGTGTTCGAA 5650
ATAAGAGTGC GAGGCAAGGG ACCAGACAGT GCTGGGGACT GGGATTATTC 5700
CGGGGACTCG CACGTGAATT GGATGCCAAG GAATAACGGT GACCAGGAAA 5750
GGCGGGGAGG CAGGATGGCG GTAGAGATTG ACGATGGTCT CAAGGACGGC 5800
GCGCAGG**T**GA AGGGGGGTGT TGGCGATGGC TGCGCCCAGG AACAAGGTGG 5850 | **var(5808):[G:0.18**]
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AGAGGAGGCG AGTGAGTGGC CAGGGAGCTG GAGATTCTGG GGTCCAGGGC 5950
AAAGATAATC TGCCCCCGA**C** TCCCAGTCTC TGATGCAAAA CCGAGTGAAC 6000 | **var(5970):[A:0.02]**
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TACCAAAAAT AGAAAAATTA ATCGGGCGCT ATGGCGGGTG **C**CTTAATCCC 6200 | **var(6191):[T:0.04]**
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TGAGACTCCG TCTCAAAAAA AAAAAAAAAA AAAAAAAAAA AGAGACTTAC 6350
TTAAGGTCTA AGATGAAAAG CAGGGCCTAC GGAGTAGCCA CGTCCGGGCC 6400
TGGTCTGGGG AGAGGGGAGG ATAGGGTCAG TGACATGGAA TCCTGACGTG 6450 E2-fw
GCCAAAGGTG CCCGGTGCCA GGAGATCATC GAC**C**CTTGGA CTAGGATGGG 6500 | **var(6484):[T:0.31]**
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CACCCCACCC CTCTGCCAGG TGGGGCAGGG GTTTCGGGCT GGTGGAGCAT 6650 | Exon 2
W G R G F G L V E H 68
GTGCTGGGAC AGGACAGCAT CCTCAATCAA TCCAACAGCA TATTCGGTTG 6700

V L G Q D S I L N Q S N S I F G C 85
CATCTTCTAC ACACTACAGC TATTGTTAGG TGAGTGGCTC CGCCCCCTCC 6750
I F Y T L Q L L L 94
CTGCCCGCCC CGCCCCGCCC CTCATCCCCC TTGGTCAGCT CAGCCCCACT 6800
CCATGCAATC TTGGTGATCC ACACAGCTGA CAGCCAGCTA GCTGCTCATC 6850 E2-rev
AC**G**GAGCGTC CTGCGGGTGG GGATGTGGGG AGGTAACTAA CAGGAGTCTT 6900 | **var(6853):[C:0.32]**
TTAATTGGTT TAAG**T**ACTGT TAGAGGCTGA AGGGCCCTTA AAGACATCCT 6950 | **var(6915):[C:0.04]**
AGGTCCCCA**G** GTTTTTTGTT TGTTGTTGTT TTGAGACAGG GTCTGGCTCT 7000 | **var(6960):[A:0.01]** |
REPEAT
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CAGTTCATGG CAACCTCTGC CTCCCTGCCC AAGGGATCCT CCCACCTTAG 7100
CCTCCCAAGC AGCTGGAATC ACAGGCGTGC ACCACTATGC CCAGCTAATT 7150
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CTCATTTAGT GCTCAGCAGG AAAATTTGTA TCTAGTCCCA TAAGAACAGA 7400
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CCATATGCCG GAGATGAGAC TATCCATTAC CACCCTTCCC AGCAGGCTCC 7500
CACGCTCCCT TTGAGTCACC CTTCCCAGCT CCAGAGAAGG CATCACTGAG 7550
GGAGGCCCAG CACCA**C**GGTC CTGGCTGACA CATGGTTCAG ACTTGGCCGA 7600 | **var(7566):[T:0.35]**
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AAGAGCTTCA GAGACCAGTC CCTTGGAGGG GACCTGTTGA AGCCTTCTTT 7700 | REPEAT
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GTGGCACAAT CATAGCTCAC TGTAACCTGG CTCAAGCGAT CCTCCTGAGT 7800
AGCTAGGACT ATAGGC**A**TGT CACTGCACCC AGCTAATTTT TTTTTTTTTT 7850 | **var(7817):[T:0.01]** |
REPEAT
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ACGCCCAGCT AATTTTTGTA TTTTT**A**GTGG AGACAGGGTT TCACCATGTT 8050 | **var(8026):[G:0.02]**
GGCTAGGATG GTCTCAATCT CTTGACCTGG TGATCCATCC GCCTTGGCCT 8100
CCCAAAGTGC TAGGATTACA GGCGTGAGTC AACCTCACCG GGCATTTTTT 8150 | REPEAT | REPEAT
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GATCTCGGCT CACTGCAACC TCCACCTCCT AGGTTCAAGC GATTCTCCAC 8250
CTTAGCCTCC CCAGCAGCTG GGATTACAGG TGCCCATCAA CACACCCGGC 8300
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TACTGGGATT ACAGGCATGA GCCACCGTGC CCAGCTGAAT TTCTAAATTT 8450 | REPEAT
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CAGCAGCTCC TGGCATCTAG GTAGTGCAGT GACATCATGG AGTGTTCGGG 8650
AGGTGGCCAG TGCCTGAAGC CCACACCGGA CCCTCTTCTG CCTTGCAGGT 8700 | Exon 3
G 95 E3-fw
TGCCTGCGGA CACGCTGGGC CTCTGTCCTG ATGCTGCTGA GCTCCCTGGT 8750
C L R T R W A S V L M L L S S L V 112
GTCTCTCGCT GGTTCTGTCT AC**C**TGGCCTG GATCCTGTTC TTCGTGCTCT 8800 | **var(8773):[T:0.11]**
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Y D F C I V C I T T Y A I N V S L 145

ATGTGGCTCA GTTTCCGGAA GGTCCAAGAA CCCCAGGGCA AGGCTAAGAG 8900
M W L S F R K V Q E P Q G K A K R 162
GCACTGA*GCC CTCAACCCAA GCCAGGCTGA CCTCATCTGC TTTGCTTTGG* 8950 | *UTR*
H 163
CATGTGAGCC TTGCCTAAGG GGGCATATCT GGGTCCCTAG AAGGCCCTAG 9000 9041G>A-mut-fw
9041G>A-wt-fw
*ATGTGGGGCT TCTAGATTAC CCCCTCCTCC TGCCATACCC G*CACATGACA 9050 | **var(9041):[A:0.38]**
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C*A*TGTGTCTG CCATGGACTG TGGTGCTGGG CCTCCCTCGG TGTTGCCTTC 9250
TCTGGAGCTG GGAAGGGTGA GTCAGAGGGA GAGTGGAGGG CCTGCTGGGA 9300 3'UTR-rev
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TGGGAGGCCA AGGCAGGAGG ATCACTTGAG GCCAGGA*GTT CCAGACCAGC* 9750 | *NOT SCANNED*
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AGCTGGCGGG AAGACTGCTT GAGCCCAGAA GGTTGAGGCT GCAGTGAGCC 9900
ATGATCACTG CACTCCAGCC TGAGCAACAG AGCAAGACCG TCTCCAAAAA 9950
AAAACAAAAA ACAAAAAAAA *ACTTGTGTTA ACGTGTTAAA CTCGTTTAAT* 10000 | REPEAT
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GAGGCTCTCA GGATTTGAAT CCACCTGGTC CATCTGGCTC CAGCA**T**CTAT 10150 | **var(10146):[A:0.01]**
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ACAGGCATGC GCCACCAGGC CTGGCTAATT TTGTATTTTT AGTAGAGACA 10350
GTGTTTCTCT ATGTTGGTCA GGCTGGTCTC GCACTCCCGA CCTCAGGTGA 10400
TCTGCCTGCT TTGGCCTCCC AAA**G**TGCTGG GATTACAGGC GTGAGCCACC 10450 | **var(10424):[A:0.05]**
ATGCCCGGCC AGCAT**C**TATA ATATATGCTA AACCTTACCC ACAGCTGCCT 10500 | **var(10466):[T:0.02]**
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GAGTCCTCTG CGCCTATGAG GAAGGTGGGC ACCGCTGTCA CCCTGTTTCC 10650 | REPEAT
CAGGAAGCCA GCAGGCCCAG GGAGATACTG GGATTTGCCA AAGGCTCACA 10700
GCGAGCAAGC AGCAGTGG**G**T GCTGGCTCCT CACCACTCAG CCTGGCACAC 10750 | **var(10719):[C:0.05]**
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GCTGCCCACG TGCCCTCCAT GGTTCCAGGA TGGCACTTTC ATGCTGGGAG 10900
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TGTTTCTCAG ACAAGGCACA GGCAGGTTAG CTCTCCCAGG CCACTCAAGT 11000 | REPEAT
AGTAAGGAAA CAGATTTAAA CCCAGACAAT TTGGCTCCA**C** GGCTAAGGCC 11050 | **var(11040):[T:0.01]** |
REPEAT
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TAGGAACTGC TGGCTAGGGC AAGCAGACAG CTGAATCCTA GCCAACTGGA 11150
GGTGGAGCAG AGGTGGAGCC AGGGAGACCC AGGTGGCCTC

2. VKORC1 Coding Sequence

```
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61  gtgctctcgc  tctacgcgct  gcacgtgaag  gcggcgcgcg  cccgggaccg
ggattaccgc
121  gcgctctgcg  acgtgggcac  cgccatcagc  tgttcgcgcg  tcttctcctc
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tcaatccaac
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301  tgggcctctg  tcctgatgct  gctgagctcc  ctggtgtctc  tcgctggttc
tgtctacctg
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gggcaaggct
481 aagaggcact ga
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VIII. REFERENCES:

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