MUTATIONAL ANALYSIS OF THE HUMAN FACTOR IX PROMOTER

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I would like to thank my parents, Dayle and Bruce Manttan for all their love and support throughout my tertiary education. Thanks Dad for correcting my grammar (shocking as it was) and attempting to understand the science 'lingo' in order to do so!. I would also like to pay tribute to my sister Suzanne, who unfortunately cannot be here today. I know you would have been proud of me. This thesis is dedicated to you.

Last but not least, thank-you Dave for all the time, effort, guidance and support that you have given me throughout the course of my M.Sc. Now I can support you!.

Although haemophilia provides for an expansive area of research, I hope that this topic will not fall to the wayside of more politically favoured or "trendy" subjects as researchers struggle to attain funding.

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Abstract

Haemophilia B is a rare congenital bleeding disorder that affects 1 in 30,000 males. It is caused by a functional deficiency in the blood coagulation protein, factor IX, which is expressed primarily within the liver. Patients suffering from the Haemophilia B Leyden phenotype show a distinct pattern of factor IX expression that is characterised by severe to moderate haemophilia within children, which gradually ameliorates after puberty. Such deficiencies in factor IX are created by mutations that occur within the -22 to +13 region of the factor IX promoter. These mutations are responsible for down-regulating factor IX transcription leading to factor IX deficiency by disrupting the binding sites of transcription factors critical for factor IX gene expression. Three specific transcription factors, C/EBP, DBP and HNF4 are thought to be required for constitutive promoter expression.

The aim of this thesis was to analyse the roles of these three transcription factors in the regulation of the factor IX promoter. The current studies were focused on two regions (-220 to -202 and +20 to +45) of the factor IX promoter which have been implicated in transcriptional activation. Reporter gene assays using the human hepatoma cell line, Alexander, were carried out on both normal and mutant promoter constructs. Recognition sites for each of the three transcription factors were disrupted by oligonucleotide-directed PCR mutagenesis. The mutated promoter inserts were subsequently inserted into the luciferase reporter gene expression vector, pGL2 Basic (Promega). These constructs were then expressed within the Alexander cell line to compare the extent of transcriptional disruption created by each mutation. EMSA studies were also used to analyse the binding ability of the HNF4 transcription factor to the -6 region of the factor IX promoter.

Mutations within the -220 to +45 region of the factor IX promoter downregulated transcription from the promoter to different extents. This suggested that each transcription factor may play a different role in regulating the factor IX promoter. An increase in promoter expression observed with mutant constructs in the presence of exogenous HNF4 confirmed previous experiments which suggested that the HNF4

transcription factor could also act as an activator of promoter expression. Furthermore, the transactivation of the promoter constructs containing a mutation within the main HNF4 site at region -15 to -30 with exogenous HNF4, indicated that a second HNF4 site may be present within the factor IX promoter.

Abbreviations

A	adenine
AR	androgen receptor
ARE	androgen receptor element
bp	base pair
BRL	Bethesda research laboratories
C	cytosine
CAT	chloroamphenicol acetyl transferase
C/EBP	CCAAT enhancer binding protein
DBP	D-site binding protein
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E.coli	*Esherichia coli*
EDTA	ethylene diamine tetra-acetate
EMSA	electrophoretic mobility shift assay
G	guanine
HNF4	hepatocyte nuclear factor 4
IL-6	interleukin-6
kb	kilobase
kDa	kilodalton
ng	nanogram
MEM	minimal essential media
MPC	magnetic particle concentrator
mRNA	messenger ribonucleic acid
NF-1	nuclear factor 1
ONPG	o-Nitrophenol B-D-Galacto-pyranoside
PBS	phosphate buffered saline

PBSE	phosphate buffered saline EDTA
PCR	polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PIC	pre-initiation complex
poly (dI-dC)	poly (dI-dC) poly (dI-dC)
RLU	relative light units
SDS	sodium dodecylsulphate
T	thymine
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TBE	Tris Boric acid EDTA
TEMED	N, N, N', N'-Tetramethylethylenediamine
TFIIA	transcription factor II A
TFIIB	transcription factor II B
TFIID	transcription factor II D
TFIIE	transcription factor II E
TFIIF	transcription factor II F
Tris	Tris-(hydroxymethyl) aminomethane
UV	ultraviolet
Xq27	chromosome X, long arm band 27

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CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND TO HAEMOPHILIA B

Haemophilia B is a hereditary bleeding disorder that affects 1 in 30 000 Caucasian males (Singer, 1978; Rubin and Forber, 1988; Gerrard *et al.*, 1993; Kay *et al.*, 1993; Kay *et al.*, 1994). This X-chromosome-linked, recessive trait (Singer, 1978; Kay *et al.*, 1994), also known as Christmas disease, arises with a deficiency of plasma factor IX (fIX) created by mutations in the factor IX gene (Singer, 1978; Rubin and Forber, 1988; Gerrard *et al.*, 1993; Kay *et al.*, 1993; Kay *et al.*, 1994). *De novo* mutations that have been identified in both the coding region and the promoter region of the factor IX gene are usually the result of single nucleotide substitutions that are thought to originate from either the misreplication of the DNA, from incorrect repair mechanisms or by physical or chemical agents acting on the DNA (Green *et al.*, 1990).

Most of the mutations within the promoter region of the factor IX gene have resulted in a phenotype called Haemophilia B Leyden (Reitsma *et al.*, 1988, 1989; Crossley *et al.*, 1990; Hirosawa *et al.*, 1990; Picketts, 1992) which is characterised by low plasma clotting activity within children (Crossley *et al.*, 1990; Picketts *et al.*, 1992). Prior to puberty, children generally suffer from moderately severe haemophilia with plasma clotting activities lower than 1% of normal plasma factor IX levels (Crossley *et al.*, 1992; Picketts *et al.*, 1992; Reitsma *et al.*, 1988). Mild or asymptomatic conditions result after puberty with factor IX in the plasma increasing to levels approximating normality at a rate of 5 % per year to reach maximum levels of 50 to 60 % of normal (Crossley *et al.*, 1990; Picketts *et al.*, 1992; Reitsma *et al.*, 1988).

1.2 HISTORICAL PERSPECTIVES OF HAEMOPHILIA B

Haemophilia B has been recognised as a hereditary haemorrhagic disorder since biblical times when patients suffering from the disease were diagnosed shortly after birth due to profuse bleeding at circumcision (Brownlee, 1987). In these cases, young

males born into families with a history of abnormal bleeding were exempted from circumcision rituals (Brownlee, 1987).

The clinical manifestations surrounding haemophilia were inexplicable for many years and patients suffering from the disease often died as a result of internal haemorrhaging (Brownlee, 1987). Before the late 1940's, patients suffering from either spontaneous bleeding episodes or bleeding as a result of physical trauma were diagnosed with classical haemophilia A, which effects 1 in 6000 males (Brownlee, 1987).

In 1947, Pavlosky reported that a second type of haemophilia was present within the human population that differed from haemophilia A (Roberts, 1993). When Pavlosky combined the bloods of two haemophiliacs, the clotting time of the admixed bloods appeared paradoxically normal. Although the two forms of haemophilia (A and B) remain clinically indistinguishable, Aggeler *et al.* (1952) and Biggs *et al.* (1952) were able to characterise, at a molecular level, haemophilia B resulting from a deficiency in factor IX levels rather than the factor VIII protein which is associated with haemophilia A. The gene responsible for the production of factor IX was also found to lie at a separate locus to the factor VIII gene on the X-chromosome (Roberts, 1993).

1.3 FACTOR IX AND ITS INVOLVEMENT IN THE BLOOD COAGULATION CASCADE

Factor IX is a 33 kDa plasma glycoprotein (Yao and Kurachi, 1992) produced in the liver (Kay *et al.*, 1993) and is involved in the blood coagulation cascade (Fig. 1) (Singer, 1978; Kay *et al.*, 1993; Kay *et al.*, 1994). Before circulating in the bloodstream, the factor IX glycoprotein undergoes post-translational modifications (Anson *et al.*, 1985; De la Salle *et al.*, 1985) which are essential for the protein to become activated by enabling the correct folding and calcium binding of the factor IX protein (Giannelli *et al.*, 1994). Such modifications include the vitamin K-dependent enzymatic modification of 12 glutamic acid residues to γ-carboxyglutamic acid, the

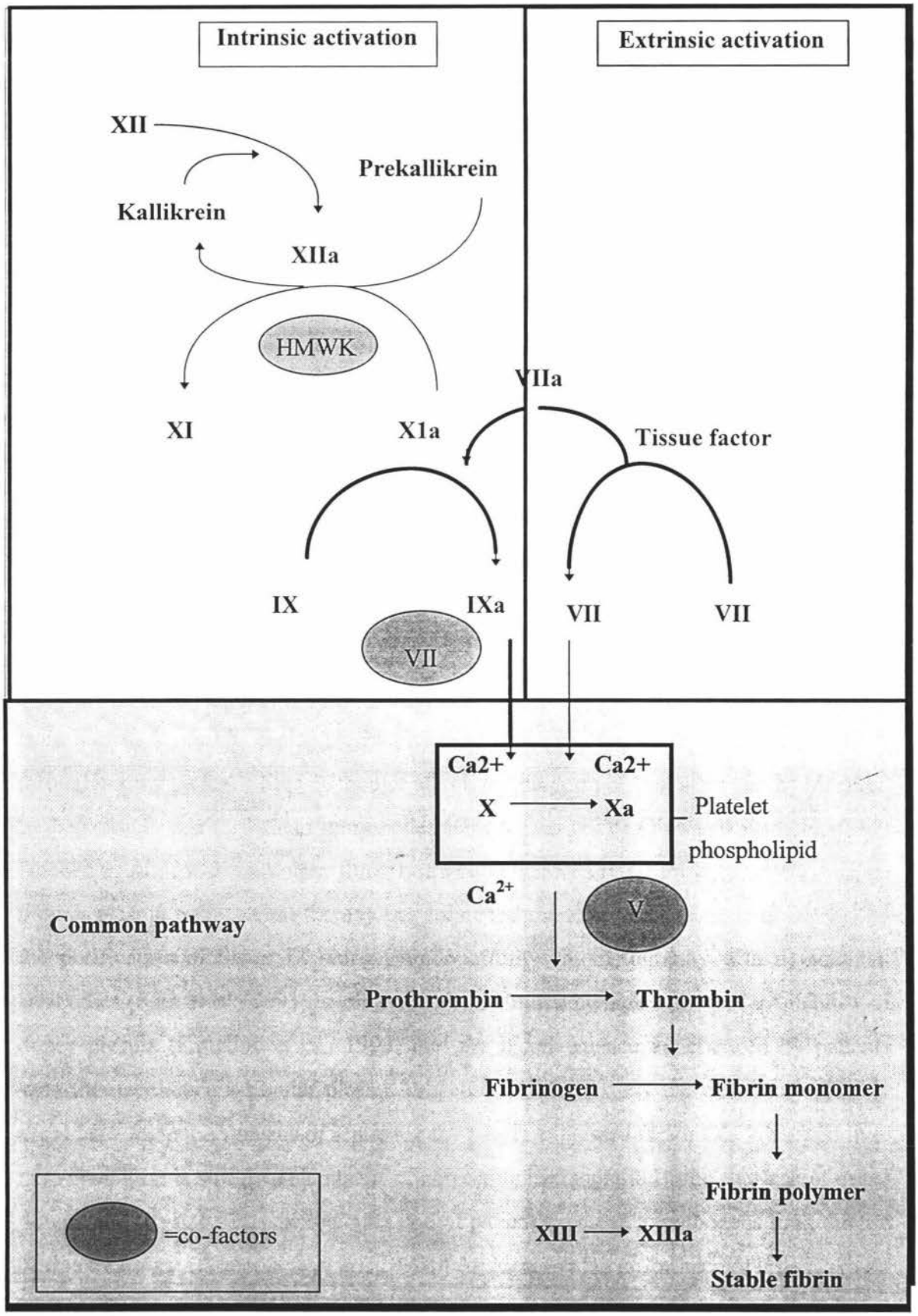


Figure 1. The blood clotting cascade. Adapted from Hoffbrand and Pettit (1993).

addition of carbohydrate residues, and the β-hydroxylation of an aspartic residue (Anson *et al.*, 1985; Busby *et al.*, 1985; De la Salle *et al.*, 1985).

After post-translational modifications, factor IX circulates as a single-chain glycoprotein of Mr=57,000 (Thompson, 1986). At this stage factor IX is found in a zymogenic (inactive) form and is activated by the onset of the blood coagulation cascade (De la Salle *et al.*, 1985). The factor IX protein is converted to an active serine protease (IXa) after proteolytic cleavage by factor XIa (Busby *et al.*, 1985). The active factor IXa protein consists of a light chain (Mr= 16,000) and a heavy chain (Mr=29,000) held together by a disulphide bond (Busby *et al.*, 1985; De la Salle *et al.*, 1985). Subsequently, factor IXa converts factor X to factor Xa in the presence of factor VIII, phospholipid and calcium ions (Busby *et al.*, 1985). Blood clotting is an extremely important homeostatic mechanism which responds to tissue damage by triggering the release of numerous clotting factors (Solomon *et al.*, 1990). Therefore an absence of factor IX would result in the inability of blood to clot thus causing patients to bruise easily, and bleed profusely from joints or wounds after minor injury (Singer, 1978).

Current treatment of haemophilia B involves the transfusion of clotting factor IX from human donor blood plasma (Palmer *et al.*, 1989) by regular intravenous injections throughout the patient's life. Although effective, this form of treatment can be hindered by hepatitis B and C, human immunodeficiency virus (HIV) (Yao and Kurachi, 1992; Hoeben *et al.*, 1995) and other thrombotic complications (Gerrard *et al.*, 1993). Even though plasma replacement therapy has improved over the years (Kay *et al.*, 1993) by the purification of factor IX using immunoaffinity chromatography with monoclonal antibodies (Kim *et al.*, 1993), such treatment is limited by high costs and availability of donor plasma (Kingdon *et al.*, 1993) and the inconvenience experienced by patients with the necessity for regular plasma infusions. Unfortunately the treatment still only provides a temporary means of replacing the poorly expressed factor IX. Although liver transplantation is still a viable means of correcting haemophilia B, the shortage of organ donors and the risk of tissue rejection and of patient exposure to pathogens limit the use of this treatment (Armentano *et al.*, 1990). An ideal treatment for haemophilia B would involve the production of genetically engineered factor IX. Research is currently being

conducted to develop a gene therapy that will stimulate the production of factor IX and therefore reduce or eliminate the need for factor IX infusions.

1.4 THE FACTOR IX GENE

The factor IX gene is a 34 kb single copy gene that is located on the subtelomeric region of the X-chromosome at position Xq.27 (Purrello *et al.*, 1985, Thompson, 1991; Roberts, 1993). Residing near to the factor IX gene is the factor VIII gene and the fragile X locus which is associated with fragile X-syndrome (Fig. 2) (Purrello *et al.*, 1985; Roberts, 1993). The factor IX gene itself comprises 8 exons that are separated by 7 introns of varying lengths (Thompson, 1991). The exons, which are highly homologous in structure, code for the structural domains represented in several blood coagulation factors that are involved in the clotting cascade. Both the introns and exons are located within the coding and 3' non-coding region of the gene (Roberts, 1993).

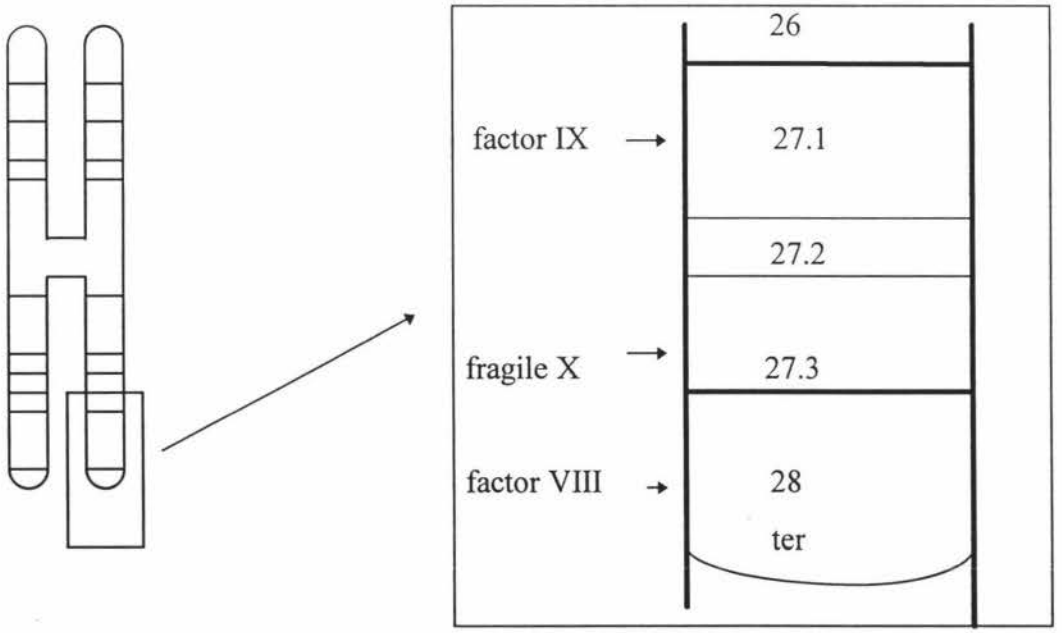


Figure 2. The location of the factor IX gene on the X-chromosome at position Xq27.1. The insert is an enlargement of the distal region of the long arm of chromosome X and shows the relation of the factor IX gene to the factor VIII gene as well as the fragile X site. Adapted from Roberts (1993).

Unlike other liver-specific genes, the factor IX gene does not contain a typical TATA box thought to be involved in transcriptional initiation. Although a TATA element has not yet been positively identified several possible locations have been suggested. These include a TCAAAT region found at nucleotides -181 of the factor IX gene; a TGTA sequence located at region -27 to -24; and a TAAA sequence located at region -43 to -40 of the factor IX gene (Anson *et al.*, 1984; Kurachi and Kurachi, 1995). The structure of the factor IX promoter reflects the co-ordinated control of gene expression by different transcription factors. The minimal factor IX promoter, which resides in a 300 bp region of the factor IX gene from nucleotides -250 to +50 relative to the transcription start point at +1 (Anson *et al.*, 1984), contains numerous elements thought to be involved in transcriptional regulation (Fig. 3). The identification of DNA binding sites by DNase I footprinting and electrophoretic mobility shift assays has assisted in understanding the molecular mechanisms by which tissue-specific transcriptional regulation is achieved.

1.5 TRANSCRIPTIONAL REGULATION OF HUMAN FACTOR IX

Mutations that occur within the minimal promoter region have been shown to disrupt the binding sites of sequence-specific transcription factors that are involved in transcription initiation from the factor IX promoter. The disablement of the binding sites by point mutations within the promoter can lead to the disruption of the initiation complex formed at the promoter which in turn can inhibit or reduce the amount of factor IX protein produced. *In vitro* studies have discovered several mutations that occur within a 40 bp region of the factor IX promoter surrounding the transcriptional start site at +1 (Reitsma *et al.*, 1988; Crossley *et al.*, 1990; Hirosawa *et al.*, 1990; Picketts *et al.*, 1992). A cluster of promoter elements required for constitutive factor IX transcription have also been located within this 40 bp (from -22 to +13) region which has subsequently been termed the Leyden-specific region (Hirosawa *et al.*, 1990; Picketts *et al.*, 1992).

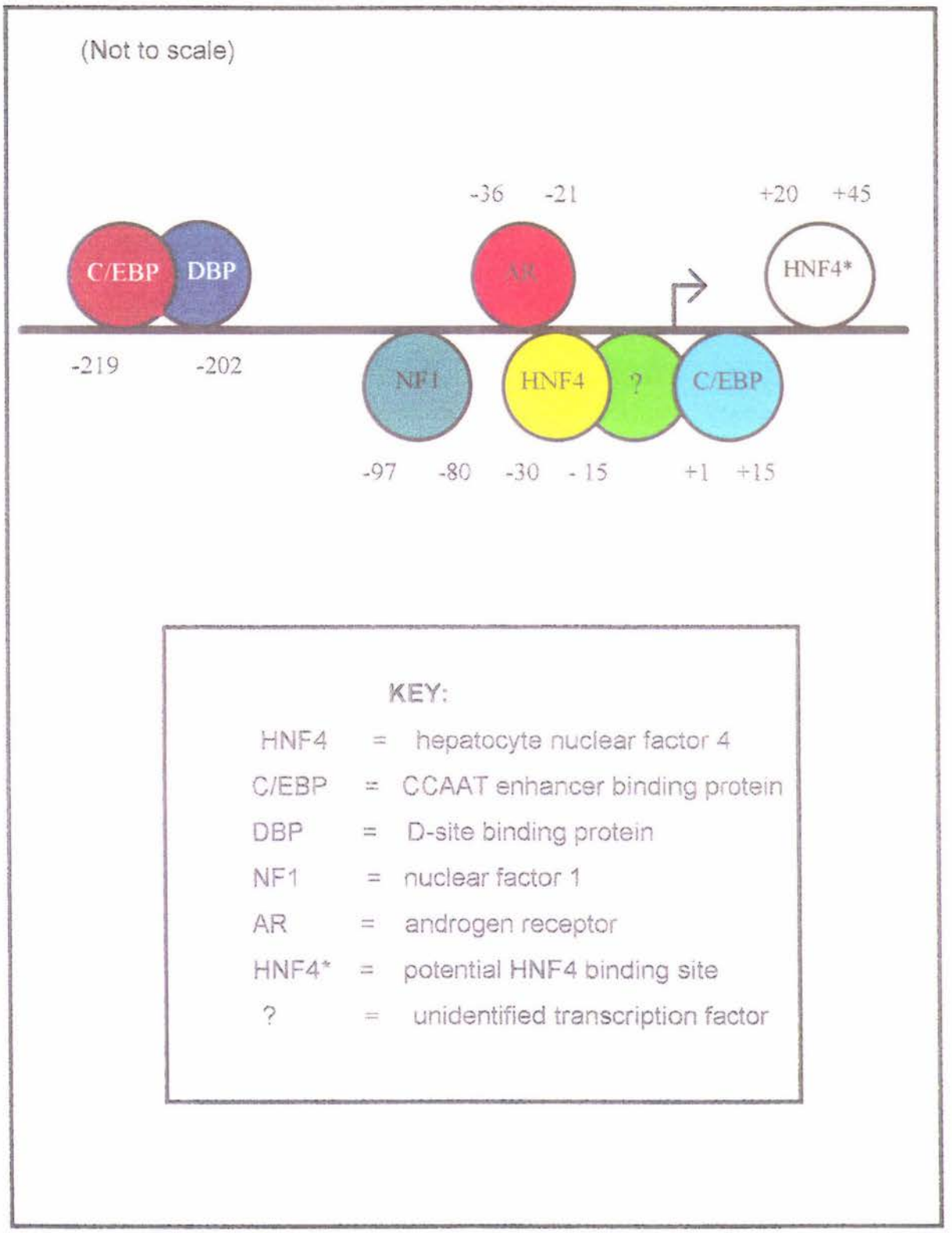


Figure 3. Schematic representation of the human factor IX promoter.

Footprinting studies have identified the binding sites for three transcription factors that interact with the promoter elements of the Leyden-specific region (Crossley and Brownlee, 1990). These correspond to the CCAAT enhancer binding protein (C/EBP) which binds to the +1 to +18 region of the promoter (Crossley and Brownlee, 1990), an unidentified transcription factor that interacts with the promoter at nucleotides +5 to +15 (Crossley and Brownlee, 1990), and a hepatic nuclear factor 4 (HNF4) transcription factor that binds to nucleotides -15 to -30 (Crossley *et al.*, 1990).

Mutations within the Leyden-specific area are responsible for down-regulating factor IX transcription leading to factor IX deficiency. The extent to which the factor IX gene is down-regulated and thus the resultant severity of haemophilia B depends upon the nature of the mutation within the promoter region (Hirosawa *et al.*, 1990). For example, a mutation that occurs within the -5 or -6 region of the factor IX promoter, disrupting the binding site of an unidentified transcription factor results in a less severe form of haemophilia B than a mutation that disrupts the binding site for the HNF4 and C/EBP transcription factors (Hirosawa *et al.*, 1990; Kurachi and Kurachi., 1995). Such mutations include the -21 T to G and -20 T to A mutations which disrupt the binding of HNF4, and the +13 A to G, +8 T to C and +6 T to A mutations which disrupt the binding of C/EBP. The variability in the severity of Haemophilia B Leyden suggests that the different transcription factors may play different roles in transcriptional initiation.

The effects of the various mutations upon the transcriptional activity from the factor IX promoter has been studied widely. The utilisation of plasmid constructs containing either the chloramphenicol acetyltransferase reporter gene or the luciferase reporter gene has made this possible, providing a rapid and quantitative means of assessing transcriptional activity after transfection within mammalian cell lines.

1.6 THE RECOVERY OF HAEMOPHILIA B LEYDEN

The molecular mechanisms responsible for the gradual amelioration in factor IX levels after puberty still remains unresolved. However, several hypotheses have been put forward to explain the recovery in the Leyden phenotype.

The discovery of an androgen responsive element (ARE) in the 5' flanking sequence of the factor IX gene implied that the phenotypic recovery may in fact be androgen-mediated (Crossley *et al.*, 1992). The ARE which is located in the factor IX promoter at nucleotides -36 to -22 was found to partially overlap the consensus sequence for the transcription factor HNF4 (Crossley *et al.*, 1992). The importance of the ARE in transcriptional regulation was further supported by the discovery of a mutation within the -26 region of the promoter which disrupted both the ARE and overlapping HNF4 binding sites (Crossley *et al.*, 1992). Unlike the Leyden phenotype, patients suffering from this mutation would fail to recover in their clinical symptoms with factor IX levels remaining abnormally low (Giannelli *et al.*, 1994). This variant form of haemophilia B was subsequently termed Haemophilia B Brandenburg (Crossley *et al.*, 1992). The inability of the factor IX levels to increase in the presence of the -26 mutation suggested that the ARE may play an important role in the developmental regulation and thus the post-pubertal recovery of haemophilia B (Naka and Brownlee, 1996).

Electrophoretic mobility shift assays performed by Crossley *et al.* (1992) demonstrated the binding of the androgen receptor (AR) to the -40 to -9 region of the factor IX promoter. The binding of the AR to the ARE within the factor IX gene is thought to be dependent on the level of testosterone present within the cell (Kurachi *et al.*, 1994; Kurachi and Kurachi, 1995). The AR, located within the cell's cytoplasm, binds to testosterone molecules in order to become activated (Kurachi *et al.*, 1994). Once bound, the AR can then interact with the ARE. As a consequence of the binding of the AR to the ARE, it has been suggested by several research groups that the promoter may either be directly transactivated (Reijnen *et al.*, 1992) or that binding may alternatively augment transcription from the promoter by further stabilising the pre-initiation complex (Picketts *et al.*, 1994). The former hypothesis would fail to explain

the similarities in factor IX levels observed between males and females (Picketts *et al.*, 1994).

In vitro studies have been performed to analyse the effects of anabolic steroids on the transcriptional activation from the factor IX promoter. The treatment of a HepG2 cell line with androgens resulted in an increase in transcriptional activation from both mutant (-6 G to A and -20 T to A) and wild-type promoter constructs (Coyle *et al.*, 1994). In similar experiments, Hirosawa *et al.* (1990) found that the rate of transcription was enhanced with increasing amounts of testosterone added into the cell line. Furthermore, haemophiliac children treated with anabolic steroids were found to produce increased levels of factor IX within their plasma (Picketts *et al.*, 1994).

Several research groups have disputed the role of the AR in the post-pubertal recovery of Haemophilia B Leyden suggesting that the modest increase in factor IX levels observed with anabolic steroids was not sufficient to explain the phenotypic recovery. Kurachi *et al.* (1993) proposed that the AR did not directly interact with the ARE, and suggested that a more widely distributed protein was responsible for binding to this element. In contrast, Picketts *et al.* (1993) suggested that a synergistic interaction between a D-site protein (DBP) and the C/EBP transcription factor at nucleotides -220 to -202 may provide an additional mechanism for the phenotypic recovery of Haemophilia B Leyden. This hypothesis was based on *in vitro* studies whereby the synergistic interaction between DBP and C/EBP proteins was able to compensate for a mutation within the -5 region of the factor IX promoter. Furthermore, the levels of DBP expressed within the mouse were found to appear in the liver only after puberty, suggesting that DBP may be involved in the expression of the factor IX gene after puberty (Reijnen *et al.*, 1992; Kurachi *et al.*, 1993). The inability of DBP to overcome a mutation within the -26 region of the promoter suggested that this mechanism alone could not be responsible for the remission of clinical symptoms. It is therefore possible that the combined action of both testosterone on the ARE and the synergistic interaction of DBP on C/EBP may be required for the post-pubertal recovery of Haemophilia B Leyden (Coyle *et al.*, 1994).

1.7 GENERAL MECHANISMS OF TRANSCRIPTION

Transcription is the process by which the genetic information held within the nucleotide sequence of DNA serves as a template for the synthesis of complementary RNA, which is in turn translated into protein (Watson *et al.*, 1992). In eukaryotic systems, transcription requires the formation of a pre-initiation complex (PIC) whereby basal initiation components including TFIIA, TFIIB, TFIID, TFIIE and TFIIF assemble together on the promoter region of the gene to be transcribed (Roeder *et al.*, 1991; Watson *et al.*, 1992).

The co-ordination of these factors required for efficient transcription is orchestrated at the start site of mRNA by RNA polymerase II, beginning with the binding of TFIID to the TATA box (Watson *et al.*, 1992). Once TFIID has bound to the TATA box, other basal components that comprise the PIC are recruited to the promoter in a sequence-specific manner (Fig. 4). This results in the stabilisation of the PIC which is subsequently recognised and bound by RNA polymerase II at the start site of transcription (Roeder, 1991). Once bound to the promoter, RNA polymerase II can then proceed to transcribe the gene of interest in a process requiring ATP hydrolysis (Watson *et al.*, 1992).

1.8 LIVER-SPECIFIC GENE EXPRESSION

Gene expression that occurs within the liver appears to be regulated primarily at the level of transcription (Roeder, 1991). In addition to the basal components that are required for PIC formation, other transcription factors are also recruited to the promoter to increase the rate of transcription (Roeder, 1991). These transcription factors, which may be either ubiquitous or liver-specific, play an important role in transcription and are, in most cases, required for constitutive promoter expression (Lai and Darnell, 1991; Aran *et al.*, 1995). For example, the transcription factor C/EBPα, which is primarily expressed within the liver, can be also located in adipose tissue, lungs and intestines (Lai and Darnell, 1991). In most liver-specific genes, the promoter region contains distinct binding sites for both the general and liver-specific transcription factors.

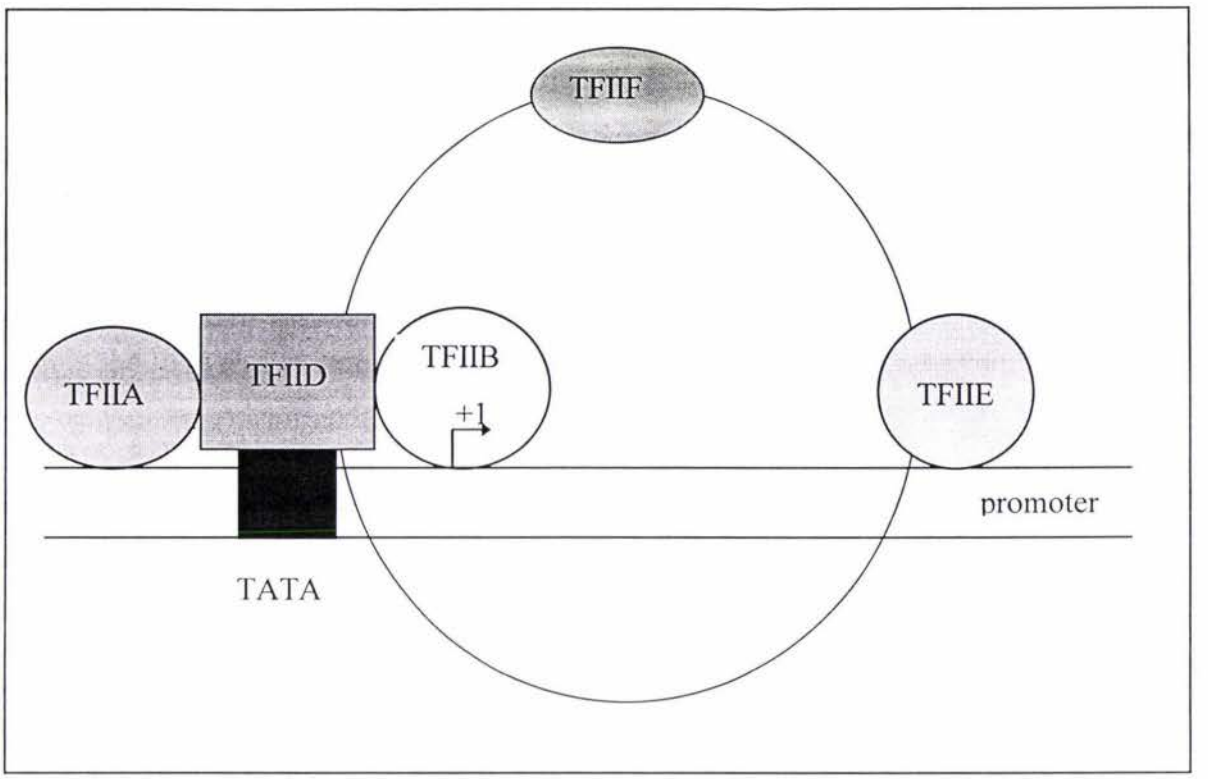


Figure 4. The pre-initiation complex (PIC) formed on the promoter. The letters A, B, D, E and F refer to the transcription factors involved in the PIC. Adapted from Roeder (1991).

The synergistic interaction between these factors and the cis-acting DNA sequences within the promoter, ensures that the gene is expressed within the correct tissue and at the right stage of development (Balsover *et al.*, 1997). The factor IX gene, which is selectively expressed within the embryonic and adult liver, provides an ideal example of how the interaction between the cis-acting DNA sequences and transcription factors can regulate cell-specific transcription as well as the timing of developmental expression.

The interaction between the basal transcriptional machinery and the transcription factors found within the hepatocyte to co-ordinate strict cell-specific transcription is not well understood (Lai and Darnell, 1991). It is possible that the transcription factors assembled at the promoter region may either interact directly or indirectly with the basal transcription machinery to alter the rate and extent of transcription (Tjian and Maniatis, 1994). Alternatively, the binding of the transcription factors to their respective DNA

elements within the promoter may result in the unwinding of chromatin structure which initially shielded the promoter from external elements. The subsequent exposure of the promoter region to basal transcriptional components may therefore permit their binding and formation of the PIC (Roeder, 1991).

The developmental hierarchy to which these transcription factors are expressed within the liver also remains enigmatic and will further aid in the understanding of the molecular mechanisms underlying gene expression (Aran *et al.*, 1995).

1.9 THE EXPERIMENTAL AIMS OF THIS THESIS

The experimental aims of this thesis were primarily based on previous *in vitro* studies performed by Franklin (1995), who characterised four mutations within the -5 and -6 region of the factor IX promoter. In these studies, the exclusion of both proximal C/EBP/DBP (region -220 to -202) and putative HNF4 (region +20 to +45) binding sites from the factor IX promoter together with specific -5 or -6 mutations resulted in a substantial decrease in the levels of factor IX transcription when expressed within the Alexander cell line. This suggested that both C/EBP and DBP transcription factors were involved in the enhancement of factor IX gene expression. Two of the four mutations analysed within the shorter construct (region -189 to +20) did however result in a higher level of transcription when compared to the wild-type construct (region -189 to +20).

Franklin (1995) proposed that the removal of the proximal C/EBP/DBP binding site may have exposed a region of the factor IX promoter to transcription factors which normally had limited access to the promoter. The recognition and binding of an unidentified transcription factor to the mutated sequence in the -5 and -6 region of the promoter may have subsequently resulted in the enhancement of gene expression.

The experimental system developed by Franklin (1995) was utilised in the current studies to determine the effect of creating point mutations within the -220 to -202 and +20 to +45 region of the factor IX promoter. The introduction of point

mutations into the binding sites of transcription factors was preferred to that of truncating these regions so as to limit the possibility of destabilising the pre-initiation complex. This would enable a more accurate assessment of the roles of the transcription factors C/EBP, DBP and HNF4 in the regulation of factor IX transcription.

Investigations into the roles of the three transcription factors were not only limited to the proximal binding sites but were also extended into other regulatory regions within the factor IX promoter including the C/EBP binding site at +5 to +15 and the HNF4 site at -15 to -30. A series of reporter gene vectors were constructed containing the factor IX promoter with point mutations within the binding sites of the respective transcription factors. Both single and double point mutations were introduced in order to determine the effect of a particular transcription factor on transcriptional regulation.

Another primary aim of this research was to analyse the possible enhancement of factor IX transcription from both wild-type and mutant Leyden promoter constructs in the presence and absence of both HNF4 and C/EBP expression plasmids in the Alexander cell line. Previous experiments with C/EBP and HNF4 expression plasmids have been restricted to the HepG2 cell line only. Therefore, luciferase reporter constructs containing various point mutations within the factor IX promoter were tested within the Alexander cell line with exogenous amounts of HNF4 or C/EBP. The extent of transactivation from the wild-type and mutant promoter constructs in the presence of these transcription factors could then be measured by a luciferase assay. Transfection efficiencies were normalised by using the external control vector pCH110 which produces β-galactosidase. The results of these experiments would subsequently indicate the importance of each transcription factor in the regulation of the factor IX promoter.

CHAPTER 2 : MATERIALS AND METHODS

2.1 MATERIALS

Minimum Essential Medium (MEM), fetal bovine serum, L-glutamine, trypsin, penicillin, streptomycin, 1 kb DNA ladder and interleukin-6 were purchased from Life Technologies Inc., MD, USA.

Luciferase Assay System™, luciferase reporter vectors, 5 x cell culture lysis reagent, and magnesium chloride were purchased from Promega Corporation, WI, USA.

Alexander cells (ATCC) were a gift from the Sir William Dunn School of Pathology, University of Oxford, UK.

The vector pTG3954 was a gift from Gareth Morgan of the Sir William Dunn School of Pathology.

The vector pCH11O was purchased from Pharmacia, LKB Biotechnology, Uppsala, Sweden.

Tissue culture flasks were provided by Sarstedt Inc., Newton, NC, USA. Tissue culture plates were purchased from either Falcon, NJ, USA, or Costar, Cambridge, MA, USA.

Cryotubes, luminometer cuvettes and cell scraper were purchased from Nunc Inc., Naperville, IL, USA. The 0.2 µl sterilisation filters were purchased from Millipore Corporation, MA, USA.

Ampicillin, tetracycline, low EEO agarose, ethidium bromide, mineral oil, TEMED, ONPG, DMSO, RNase, SDS and lysozyme were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Restriction endonucleases and their buffers, T4 DNA ligase and buffer, proteinase K, ammonium persulphate, oligonucleotides, dATP, dCTP, dTTP, and dGTP were purchased from Life Technologies Inc., MD, USA.

The *E. coli* bacterial strain XL-1, *Pfu* DNA polymerase and buffer were purchased from Stratagene, La Jolla, CA, USA.

Taq DNA polymerase and buffer and the Wizard Maxiprep™ DNA Purification System were purchased from Promega Corporation, WI, USA.

Radioisotopes were purchased from Dupont Research Products, Boston, MA, USA.

40 % Acrylamide/Bis solution (29:1, 3.3 % C and 19:1, 5 % C) was obtained from Biorad Laboratories, CA, USA.

Poly (dI-dC) and dNTPs were purchased from Pharmacia, LKB, Technology, Upssala, Sweden.

Sequenase® Version 2 and associated reagents were supplied by US Biochemical Corporation, Cleveland, Ohio, USA.

Dynabeads™ M-280 Streptavidin were purchased from Dynal A.S., Oslo, Norway.

The ion exchange chromatography paper, DE-81 and Whatman Filter paper were purchased from Whatman International Ltd., Maidstone, England.

Photographic developing and fixing solution were purchased from Eastman Kodak, NY, USA. X-ray film was supplied by Fuji Photo Film Company Ltd., Japan.

2.2 METHODS

2.2.1 Introduction

2.2.1.1 An overview of cell culture methodology

The human hepatoma cell line, Alexander, was maintained using minimum essential media containing 10 % of fetal calf serum (FCS), 1 % non-essential amino acids and antibiotics. The cells were passaged regularly in order to maintain cellular stocks, allow further growth or prepare for transient transfections. The procedures involved in tissue culture are outlined in Figure 5.

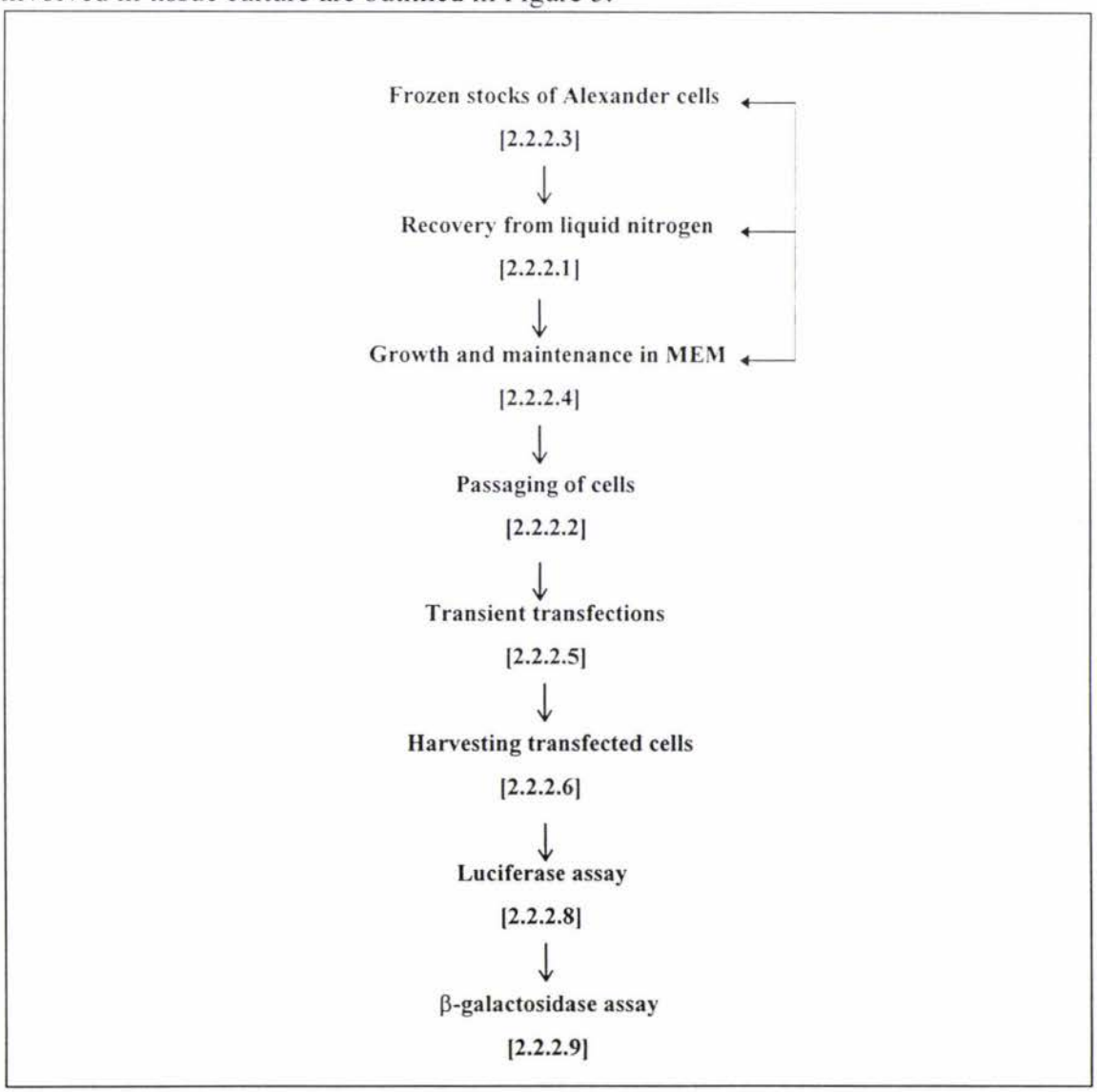


Figure 5. An outline of the procedures utilised in the preparation and maintenance of the hepatoma cell line, Alexander. The numbers in brackets refer to the appropriate chapter heading.

2.2.2 Cell culture and transfection

2.2.2.1 Recovery of cells from liquid nitrogen

T80 tissue culture flasks were prepared each containing 12 ml of complete MEM. Stocks of Alexander cells frozen within cryotubes were thawed quickly at 37°C (2 minutes) and immersed into 70 % ethanol for a further 2 minutes to sterilise the outside of the cryotube. The cells within the cryotube were then transferred to a 15 ml centrifuge tube containing 3 ml of complete MEM. The tubes were then centrifuged (250 x g) for 3 minutes with the supernatant being discarded and cell pellet resuspended into a further 3 ml of complete MEM. Cells were dispersed into T80 flasks for growth and were placed back into the incubator at 37°C until they reached confluence.

2.2.2.2 Passaging of cells

Microscopic examination was used to determine the appropriate stage for passaging cells. This stage comprised a monolayer of cells that had reached ~80 % confluency. Subsequently, the monolayer was broken up and distributed into new flasks or transfection wells. The old medium was removed from tissue culture flasks, and the cells were detached from the bottom of flasks with the addition of 3 ml of PBSE [0.14 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4.7H_2O$, 1.5 mM KH_2PO_4 (pH 7.2), and 0.5 mM EDTA] with 0.25 % trypsin. The flasks were gently rocked to distribute the trypsin evenly over the cells. Plates were then left for a period of 5 minutes at room temperature to allow the trypsin to dislodge cells. However, it was important that the trypsin was not left on the cells for an extended period because of its toxic nature. Microscopic examination of the plates helped in determining whether cells had been dislodged from the plates; *i.e* cells became singular and rounded in appearance. Cells were then collected into 15 ml centrifuge tubes containing 5 ml of fresh, complete MEM and pelleted by centrifugation at 250 x g for 3 minutes. After resuspension in another 3 ml of complete MEM, cells were either seeded into flasks or tissue culture plates depending on whether the cells were to be transfected or regrown.

2.2.2.3 Maintenance of frozen stocks

Frozen stocks of the Alexander cell line were prepared by the passaging of cells and their subsequent resuspension in 200 µl of DMSO and 1.8 ml of fetal calf serum. Cells were dispersed into 1.5 ml cryotubes and quickly wrapped in tissue paper before being placed in the -70°C freezer overnight. After 24 hours the cryotubes were transferred to liquid nitrogen for long-term storage.

2.2.2.4 Maintenance of cells

The human hepatoma cell line, Alexander, was established from a stock stored in liquid nitrogen, and was maintained within T80 transfection flasks (Starstedt Inc, USA) each containing 12 ml of complete media. The powdered form of MEM (and non-essential amino acids) required the addition of sterile water and sodium bicarbonate (2.2 g/l), and was adjusted to a pH of 6.6 to 6.8 before being membrane filtered through a 0.2 µm Acrocap™ filter (Gelman Sciences, MI, USA). Prior to use, the MEM was supplemented with 10 % fetal calf serum, penicillin (500 U/ml), streptomycin (500 mg/ml) and L-glutamine (2 mM) to make complete MEM. Alexander cells were incubated at 37°C in a 5 % CO_2 humidified atmosphere to allow growth. The medium was changed every 2 to 3 days to remove dead cells and debris. Cells were examined microscopically for atypical morphology (*e.g.* structural distortion, large vacuoles and more than one nucleus), cytotoxicity (*e.g.* bacterial infections, fungal growth) and the percentage of confluence. All cell manipulations were performed within a laminar flow hood (Email Westinghouse Pty Ltd., Australia) to maintain a sterile environment.

2.2.2.5 Transient transfections

Transient transfections of the human hepatoma cell line, Alexander, were carried out by the standard calcium-phosphate precipitation methodology outlined by Ausubel *et al.* (1989).

All transient transfections were carried out under sterile conditions within the laminar flow hood to limit the chances of cell infection and contamination. Buffers A [0.5 M $CaCl_2$, 0.1 M Hepes (pH 7.05 to 7.12)] and B [0.28 M NaCl, 0.05 M Hepes, 0.75 mM NaH_2PO_4, 0.75 mM Na_2HPO_4 (pH 7.05 to 7.12)], which were used in the transient transfections, had previously been filter sterilised (0.2 μm filter) and tested in their appropriate quantities for precipitate formation. Two different sized tissue culture plates were used during the course of this study. Initial transient transfections were carried out in single 6 cm diameter tissue culture plates (Falcon, USA). However, the purchase of the smaller tissue culture multiwell plates (Costar, USA) proved to be more efficient in saving both time and reagents.

Alexander cells were passaged and seeded into either 6 cm tissue culture dishes or 15 mm cell wells the day before transfections were carried out to allow 20 to 30 % confluence in each dish. Cell densities were examined under the microscope in order to assess whether the cell number was appropriate for efficient transfection. Two hours prior to transfection, the medium from the tissue culture wells was removed and replaced with fresh complete MEM. In the meantime, all plasmids and buffers were thawed and warmed to room temperature, and the plasmid DNA diluted to a concentration of 1 mg/ml or 0.5 mg/ml (6 cm plates and 15 mm cell wells, respectively) with sterile TE buffer [10 mM Tris.HCl, 1 mM EDTA; pH 7.5]. For the larger 6 cm tissue culture plates, 10 μg of reporter plasmid DNA and 5 μg of the β-galactosidase expression vector (pCH110) were used in each transfection. The smaller 15 mm plates required one fifth of the plasmid DNA and the reagents used in a typical transfection *i.e* 2 μg of plasmid DNA and 1 μg of pCH110.

In a typical transfection, the plasmid DNA was further diluted to a volume of 240 μl in sterile water followed by the addition of 240 μl of Buffer A. This was followed by the addition of 240 μl of Buffer B and vigorous vortexing of the reaction mixtures for 3 minutes. The DNA-calcium phosphate suspension was incubated at room temperature for 15 minutes to allow the precipitate to form. The precipitate was then added dropwise to the medium which was swirled to evenly distribute the precipitate and avoid localised toxicity to the cells. The cell plates were returned to the

37°C incubator for a further 16 to 24 hours before the cells were washed twice with 3 ml of PBS buffer [0.14 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4.7H_2O$, 1.5 mM KH_2PO_4; pH 7.2] and replenished with fresh complete MEM. Cells were left for a further 16 to 24 hours in the 37°C incubator before being harvested. Transient transfections were carried out until at least three independent experiments gave reproducible results.

2.2.2.6 Harvesting Cells

The medium containing the DNA precipitate was discarded 16 to 24 hours after transfection. The cells were briefly washed twice with 3 ml of PBS to remove any remaining precipitate and medium. The cells were harvested by the addition of 250 µl or 100 µl of 1 x cell culture lysis reagent (Promega, USA) in the 6 cm and 15 mm tissue culture plates respectively. The plates were left to stand for 15 minutes at room temperature before the cell lysates were removed using a cell scraper, and collected into 1.5 ml microcentrifuge tubes, which were subsequently spun down to remove any cellular debris. The resultant supernatant was transferred into a clean microcentrifuge tube.

2.2.2.7 Addition of interleukin-6

The reconstitution of interleukin-6 was carried out according to the manufacturer's instructions. Interleukin-6 (IL-6) was supplied in a lyophilized form and stored desiccated at -20°C. IL-6 was reconstituted in sterile PBS buffer to 100 ng/µl, and prior to use IL-6, was diluted to a concentration of 1 ng/µl in PBS buffer containing 0.1 % fetal calf serum. In order to achieve maximum efficiency with IL-6, it was important to avoid repetitive freeze-thaw cycles. IL-6 was added in appropriate concentrations (Chapter 3) to Alexander cells 16 to 24 hours after the cells had been transiently transfected and washed with PBS buffer to remove any remaining precipitate.

2.2.2.8 Luciferase assay

The Alexander cells transfected with luciferase reporter gene constructs were harvested and assayed for luciferase activity. The luciferase activity from each construct was determined by mixing 20 µl of cell extract with 100 µl of luciferase assay reagent from the Luciferase Assay System™ (Promega, USA). The photon emittance created from the enzymatic reaction was measured in a Bio-Orbit 1253 luminometer. The luciferase assays were performed immediately after the cells had been harvested with the luciferase reagents equilibrated to room temperature, so that optimal activity could be attained.

2.2.2.9 β-galactosidase assay

The β-galactosidase method developed by Herbomel *et al.* (1984) was used to normalise for any variance that occurred in transfection and harvesting efficiencies. The β-galactosidase activity from each cell lysate was determined by the addition of 400 µl of β-galactosidase buffer [60 mM NaH_2PO_4, 40 mM Na_2PO_4, 10 mM KCl, 1 mM $MgCl_2$] and 200 µl of 2 mg/ml O-nitrophenyl-β-galactoside (ONPG) in 60 mM NaH_2PO_4 and 40 mM Na_2HPO_4 to 20 µl of cell lysate solution. The cell extracts were incubated for 2 hours at 37°C before the enzymatic reaction was stopped with the addition of 500 µl of 1 M Na_2CO_3. The absorbance of each cell lysate reaction was measured spectrophotometrically at 420 nm.

2.2.3 Construction of the luciferase reporter vectors

2.2.3.1 An overview:

Two expression vectors, pGL2 Basic and pGL2 Enhancer were used in reporter gene construct preparation. Mutated factor IX promoter regions (-220 to +45) were ligated into the reporter vectors and the constructs sequenced to check for any abnormalities during manipulations. All plasmids were prepared in large quantities

before being utilised in transient transfections. The protocols involved in preparing plasmids are outlined in Fig. 6.

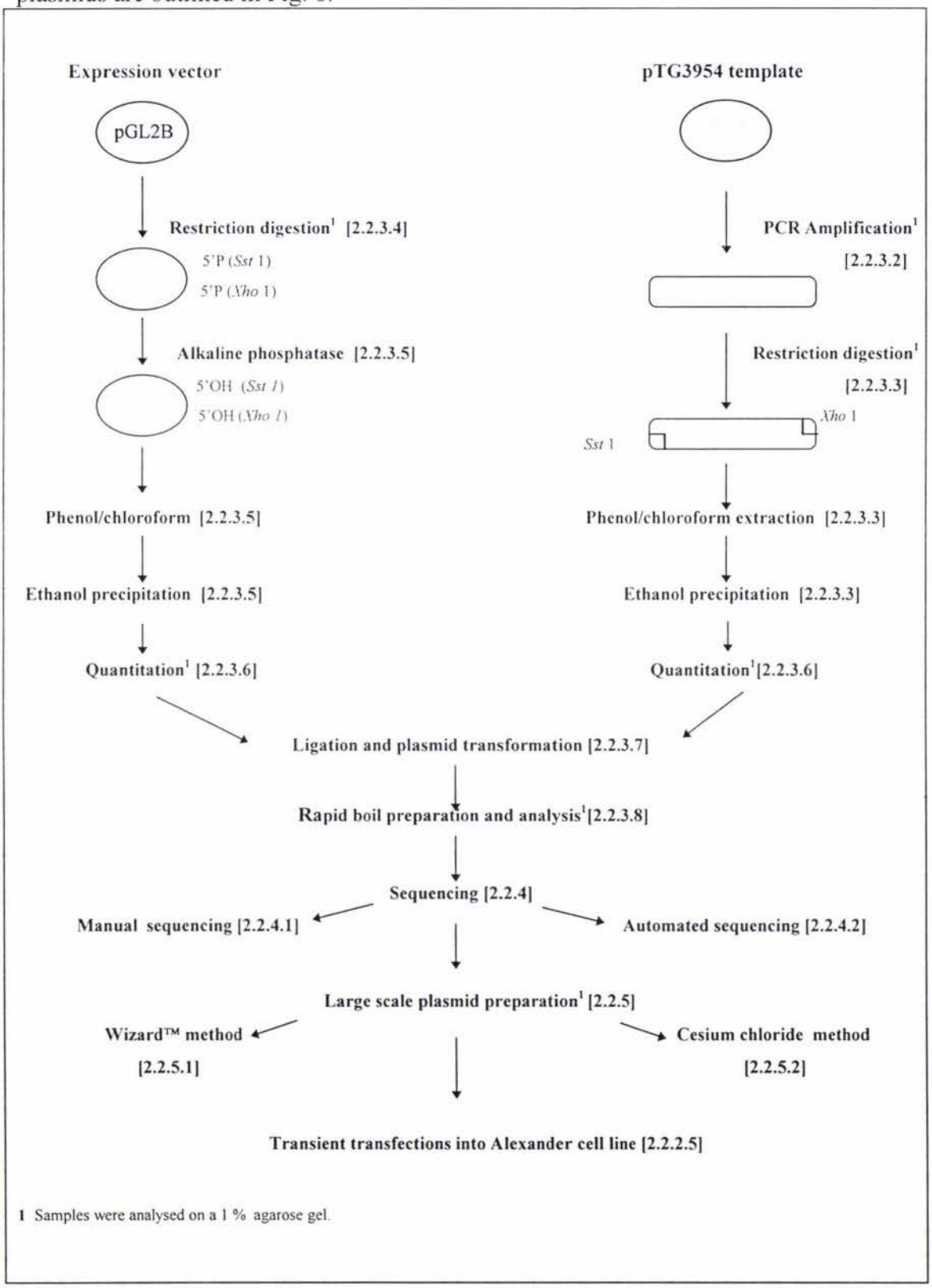


Figure 6. An outline of the procedures used for the preparation of reporter gene constructs. The numbers in brackets refer to the appropriate chapter heading.

2.2.3.2 PCR amplification of the factor IX minimal promoter

The -220 to +45 region of the factor IX promoter was amplified by PCR from the template pTG-3954 (Appendix I) using the high fidelity enzyme *Pfu* DNA polymerase, following the protocols recommended by Cetus corporation Ltd. Each 50 µl reaction contained 1-10 ng of template DNA, 250 ng of oligonucleotide primer, 300 µM of dinucleotide triphosphates (dNTP), 2.5 U of *Pfu* DNA polymerase in the corresponding *Pfu* polymerase buffer. The contents of each PCR tube were overlaid with mineral oil to prevent evaporation. The thermal cycling parameters for amplification comprised 30 cycles of denaturation in which samples were heated to 95°C for 1 minute followed by cooling to an annealing temperature of 60°C for 1 minute and finally an extension stage at 72°C (1 minute). Five percent of each PCR reaction mixture was analysed by electrophoresis in a 1 % agarose gel to check for any contamination or band abnormalities.

2.2.3.3 Restriction digestion of PCR products

PCR products were digested in a total volume of 50 µl containing 45 µl of PCR reaction, 5 µl of React® buffer, 1-10 U of restriction enzyme and sterile water. All reaction tubes were incubated for an hour at 37°C before 5 µl of the total volume of restriction digestion was analysed on a 1 % agarose gel to confirm the bands identity. The digested PCR products were subsequently purified by phenol chloroform extraction and concentrated by the ethanol precipitation method as described by Sambrook *et al.* (1989).

2.2.3.4 Restriction digestion of the vector

Vector DNA was linearised by digestion with restriction enzymes in a total volume of 50 µl, containing 1 µg of expression vector, 1-10 U of restriction enzyme, 5 µl of React® buffer and sterile water up to 50 µl. The reaction tubes were mixed and

incubated at 37°C for an hour. Five µl of the total reaction volume were analysed on a 1 % agarose gel to check for complete digestion.

2.2.3.5 Removal of the 5' phosphate groups

The cleaved vector DNA was treated with calf alkaline phosphatase to prevent the re-ligation of the expression vector. In a 1.5 ml microcentrifuge tube, 1 µl of calf alkaline phosphatase was added to the restriction digest and incubated at 37°C for 15 minutes. After the incubation period, 2 µl of proteinase K (10 mg/ml) and 2 µl of 20 % SDS were added, and the solution incubated for an additional hour at 37°C. The vector DNA was then purified by phenol/chloroform extraction and concentrated by ethanol precipitation as described by Sambrook *et al.* (1989).

2.2.3.6 Quantitation

The amount of purified product was quantitated by electrophoresis on a 1 % agarose gel alongside quantitation standards 100 ng/ 5 µl, 50 ng/ 5 µl, 20 ng/ 5 µl and 10 ng/ 5 µl. A visual comparison between the plasmid band and quantitation standards on a 1 % agarose gel gave an estimate of the plasmid concentration.

2.2.3.7 Ligations and transformation

Ligation reactions were carried out as described by Sambrook *et al.* (1989) in a total volume of 10 µl using a vector to insert ratio of 1:3. All ligation reactions involved cohesive ends therefore the ligation reactions were incubated at 16°C overnight in a waterbath to ensure complete annealing of the insert to the vector. Plasmid transformation into the bacterial strain *E.coli* XL-1 Blue was carried out using 5 µl of ligation reaction and competent cells prepared by the $CaCl_2$ method as described by Sambrook *et al.* (1989).

2.2.3.8 Rapid boil preparation and analysis

Plasmid DNA was isolated from *E.coli* by the rapid boil method as described by Holmes and Quigley (1981). All plasmids were analysed by restriction endonuclease digestion to confirm their identity.

2.2.3.9 Glycerol stocks

E.coli stocks in glycerol were prepared by transferring 800 µl from an overnight culture to a sterile 1.5 ml cryotube containing 200 µl of autoclaved 100 % glycerol. Bacterial stocks were stored in the -70°C freezer.

2.2.4 DNA sequence analysis of the factor IX promoter

2.2.4.1 Preparation of templates for manual sequencing

DNA sequence analysis was determined by the chain-termination DNA sequencing method developed by Sanger *et al.* (1977). Sequencing templates were prepared by PCR using one primer which was biotinylated at the 5'-end and one normal primer.

Single-stranded templates were prepared using streptavidin paramagnetic M-280 dynabeads™. PCR products were analysed by electrophoresis on a 1 % agarose gel to ensure that only one band had been produced. After this analysis, 40 µl of the PCR products were mixed with 40 µl of streptavidin paramagnetic M-280 dynabeads ™ that had been previously washed in 80 µl of 1 x binding and washing buffer [5 mM Tris.HCl (pH 7.5), 0.5 mM EDTA, 1 M NaOH] and resuspended in 160 µl of 2 x binding and washing buffer. Constant resuspension was required for 15 minutes before the beads were magnetically separated from the supernatant using a magnetic particle concentrator (MPC). A further 40 µl of 1 x binding and washing buffer were added to the beads before the supernatant was magnetically separated, and the beads resuspended in 8 µl of 0.1 M NaOH. After a 10 minute period at room temperature, the supernatant was

removed and the beads sequentially washed with 50 µl of 0.1 M NaOH, 40 µl of 1 x binding and washing buffer and 50 µl of TE buffer (pH 8.0). The supernatant was removed using the MPC and the beads were subsequently resuspended in 7 µl of sterile deionised water.

The resuspended beads containing the single-stranded DNA template were sequenced using Sequenase®Version 2.0 (9th ED., USB, USA) according to the manufacturer's instructions. A 3 µl aliquot of each sample was loaded onto a 6 % denaturing polyacrylamide gel in 1 x TBE buffer [0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA; pH 8.0 containing 7 M Urea] which was prepared according to the method outlined by Sambrook *et al.* (1989).

Electrophoresis was carried out at 1500 V constant voltage (65 W and 45 mA) until the bromophenol blue had reached the bottom of the sequencing plates. This represented a sufficient distance for the ~200 bp that was required for analysis. The gel was then placed in a fixing solution [10 % ethanol/acetic acid] for 10 to 15 minutes before being dried under vacuum onto 3 MM blotting paper. The gel was autoradiographed overnight at room temperature before being developed and analysed manually.

2.2.4.2 Preparation of sequencing templates for automated sequencing

A 1 ml overnight culture was pelleted by centrifugation (12000 r.p.m, 1 minute, room temperature) and resuspended in 200 µl Glucose/Tris/EDTA (GTE) buffer [50 mM Glucose, 25 mM Tris (pH 8.0), 10 mM EDTA, pH 8.0]. A 300 µl solution of freshly prepared 0.2 M NaOH/1 % SDS was added to each mixture and left on ice for 5 minutes. The solution was neutralised by the addition of 300 µl of 3 M potassium acetate (pH 4.8), and then left on ice for a further 5 minutes. The tubes were then centrifuged for 10 minutes (12000 r.p.m, room temperature) and the supernatant transferred into a clean microcentrifuge tube. The volume of the supernatant was measured and RNase (10 mg/ml) was added to give a final concentration of 20 mg/ml.

The tubes were incubated at 37°C for 20 minutes, after which the aqueous phase was extracted twice with 400 µl of chloroform, and placed into a clean tube. An equal volume of 100 % isopropanol was added to the aqueous phase and centrifuged (12000 r.p.m, 10 minutes, room temperature). After discarding the supernatant, the resultant pellet was washed with 500 µl of 70 % ethanol and dried by aeration. The pellet was subsequently dissolved in 32 µl of sterile deionised water prior to the addition of 8 µl of 4 M NaCl and 40 µl of sterile 13 % PEG. The solution was then incubated on ice for 20 minutes and the DNA repelleted by centrifugation (12000 r.p.m, 15 minutes, 4°C). This pellet was washed with 500 µl 70 % ethanol and was left to dry. The DNA pellet was then resuspended in 20 µl sterile deionised water. Prior to automated sequencing, all plasmids were analysed on a 1 % agarose gel to quantitate the DNA within each sample and to check for contamination or band abnormalities.

2.2.5 Large scale preparations of the factor IX clones

Two different methods of large-scale plasmid preparation were employed during the course of this work: (1) Wizard maxiprep preparation and purification method - which involves an alkaline lysis procedure as well as a resin to isolate and purify DNA; (2) Cesium chloride plasmid preparation - whereby the plasmid DNA is separated by the means of a density gradient.

2.2.5.1 Wizard Maxiprep Preparation and Purification Method

Plasmids were prepared and purified on a large scale using the Wizard™ Maxipreps DNA Purification System following the standard procedure as described by Promega's Technical Bulletin (139). This method involved the purification of plasmids from a cleared lysate and a proprietary silica-based anion exchange resin to give a high yield of purified DNA.

To determine the concentration of plasmid present within each preparation, plasmid solutions were diluted (1/50 dilution factor) in TE buffer (pH 8.0). The plasmid

DNA was then quantitated spectrophotometrically by measuring the absorbance at 260 and 280 nm.

2.2.5.2 *Cesium chloride plasmid preparation*

Plasmids were prepared and purified using the Cesium chloride method as described by Sambrook *et al.* (1989). This method involved the purification of plasmids from cleared lysates of bacterial cells by centrifugation in gradients of CsCl-ethidium bromide.

2.2.6 Electrophoretic mobility shift assay (EMSA)

2.2.6.1 *Preparation of radioactive DNA probes for electrophoretic mobility shift assay*

100 ng of an oligonucleotide was end-labelled in a 10 µl total reaction volume containing 3 µl of [(γ-^{32}P)]-rATP, 1 µl of 10 x T4 kinase buffer [35 mM Tris.HCl (pH 7.6), 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM β-mercaptoethanol] and 10 U (1 µl) of T4 polynucleotide kinase. Reagents were mixed and incubated at 37°C for 45 minutes. This was followed by the addition of 600 ng of cold complementary unlabelled oligonucleotide to the reaction mixture, together with 2.5 µl of 1 M KCl and sterile water to a total volume of 50 µl. The mixtures were heated to 95°C for 5 minutes and slowly cooled to ensure annealing was complete. Prior to gel purification, 50 µl of 2 x gel shift buffer [40 mM Tris (pH 7.6), 16 % Ficoll, 100 mM KCl, 0.4 mM EDTA, 1 mM DTT] were added to the annealed oligonucleotides.

Gel purification was performed by non-denaturing electrophoresis using a 10 % polyacrylamide gel in 1 x TBE buffer [0.09 M Tris, 0.09 M boric acid, 0.02 M EDTA; pH 8.0] at 30 W. Electrophoretic progress was monitored by the presence of DNA loading dye (5 µl) [0.25 % Bromophenol blue and 40 % (w/v) sucrose] that was added in a control lane. Electrophoresis was continued until the bromophenol blue had reached 10 cm from the bottom of the 37 cm gel plates. The gel was subsequently

wrapped in gladwrap and exposed to X-ray film for ~1 minute. The band was located and excised from the gel by matching the developed film to its relative position on the gel. The labelled oligonucleotide was eluted from the gel by incubation overnight in 200 µl of 50 mM KCl. The supernatant was collected after centrifugation at 13000 r.p.m for 1 minute. The amount of radioactivity present within a 1 µl aliquot of the labelled oligonucleotide was determined by Cerenkov counting using a Beckman LS8000 scintillation counter. An activity of at least 10000 cpm/µl was required for EMSAs to be informative. The radioactive oligonucleotide was stored at 4°C and used over a period of 2 to 3 weeks.

2.2.6.2 Electrophoretic mobility shift assays (EMSA)

Electrophoretic mobility shift assays were carried out in a 20 µl reaction volume containing 10 µl of 2 x EMSA buffer [40 mM Tris (pH 7.8), 10 % Ficoll, 50 mM KCl, 0.4 mM EDTA and 1.0 mM DTT], 1 µg of the non-specific competitor, poly (dI-dC), and 1 µl of Cos cell extract in the presence or absence of the unlabelled competitor oligonucleotides. The poly (dI-dC) was dissolved in 50 mM KCl at a concentration of 1 µg/µl before being sonicated and stored at -20°C. The reaction mixtures were incubated on ice for 10 minutes before the addition of 1 µl of radioactive oligonucleotide (10000 to 20000 cpm). After 15 minutes at room temperature, half of the total reaction mixture was loaded on to a 4 % non-denaturing gel in 0.25 x TBE buffer. A 5 µl aliquot of DNA loading dye was added to one lane to enable electrophoretic progress to be monitored. Electrophoresis was carried out at 200 V constant voltage for ~2 hours until the bromophenol blue from the control lane had run 3/4 of the way down the 15 cm gel plates.

The gel was then dried for 15 minutes under a vacuum onto DE-81 chromatography paper before being autoradiographed overnight at -70°C. The ion-exchange chromatography paper, DE-81, was utilised in these experiments to prevent the loss of free probe. For the competition experiments, 5 ng, 50 ng and 100 ng (10, 100 and 200 fold in excess respectively) of double-stranded competitor oligonucleotides were added to the reaction mixture prior to the addition of the labelled oligonucleotide.

2.2.6.3 Cos cell extract preparation

Whole cell extracts were prepared from Cos cells as a source of transcription factor. Cos cells were maintained in DMEM containing 10 % fetal calf serum and antibiotics. The cells were seeded into 150 mm dishes to produce ~60 % confluence and transfected with 45 μg of expression plasmid using the calcium phosphate co-precipitation method. The cells were left in a 37°C incubator (5 % CO_2) before they were washed with PBS and harvested in TEN buffer [40 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.15 M NaCl]. The cells were pelleted and resuspended in 300 μl of extraction buffer [40 mM Hepes (pH 7.9), 0.4 M KCl, 1 mM DTT, 10 % glycerol, 0.1 mM PMSF, 0.1 % aprotinin]. The cells were disrupted by three freeze-thaw cycles and centrifuged for 5 minutes at 14000 rpm, at 4°C. The supernatants were dispersed in 30 μl aliquots into 1.5 ml cryotubes, snap-frozen in liquid nitrogen and stored at -80°C.

CHAPTER THREE: RESULTS

3.1 CELL CULTURE AND TRANSFECTION

3.1.1 Introduction

Alexander cells (ATCC) were initially established from frozen stocks stored in liquid nitrogen and were maintained in minimal essential media and 1 % non-essential amino acids. Cells grown from liquid nitrogen stocks exhibited an initial lag phase of growth when seeded into tissue culture flasks. The lag phase of growth lasted from 1 to 3 days depending upon how dilute the initial innoculum was. After the cells had adjusted to their new environment and conditioned the media the number of cells present within the flasks began to increase exponentially. The media were exchanged every 2 to 3 days to supply the required nutrients to the cells and to remove any build-up of acids and other waste-products that may have accumulated. Before long the cell number began to plateau and a monolayer of cells was formed. The Alexander cell line required passaging every 2 to 3 days to maintain optimal conditions. In order to assess the stage of cell growth and the percentage of cell confluence *i.e* suitability for passaging/transfections, the cells were examined regularly by microscope. Microscopic examination was also used to check for any cytotoxicity *i.e* bacterial infection or contamination due to non-sterile technique, and for the discovery of mutations within the cell line. Mutations in the cell line may originate and escalate from over-passaging and the mutated cell line may eventually outgrow and replace the existing healthy cells. Mutations that arise within the cell population are identified by their atypical morphology.

Several parameters in the maintenance of cells have been noticed to affect the successfulness of transfection experiments. These include:

(1) the number of times the cell line has been passaged,
(2) the density of the cells used to seed the transfection experiment, and,
(3) the extent of confluency in transfection experiments (Glover and Hames, 1995).

It is important that cells do not become overly dense when growing as this may effect their overall viability and subsequent efficiency in transfection. Other factors that have also been noted to effect cellular viability and/or plating efficiencies include the over-trypsinization of cells during their passaging, and the differences in batches of fetal calf serum which is incorporated into the media. These parameters were taken into consideration when maintaining the Alexander cell line in order to maximize the levels of efficiency to be obtained throughout the course of this study.

In these studies, transient transfections were performed using the calcium phosphate precipitation method. The calcium-phosphate technique of DNA intake has been used widely and is one of many methods utilised for the uptake of DNA into mammalian cell lines. It involves the formation of a calcium-phosphate co-precipitate when exogenous DNA is mixed with calcium chloride and added to a solution containing phosphate ions thus resulting in the uptake of DNA. However different cell lines vary in their ability to take up and express exogenous DNA. For example, the two human hepatoma cell lines, HepG2 and Alexander, express different levels of the same protein in transient transfections (Franklin, 1995). The different levels of transcription factors present within a cell line must therefore be taken into consideration when analysing the overall effects of promoter mutations within a cell line. The Alexander cell line was chosen for these experiments because of their ease of growth and hardiness.

3.2 REPORTER GENE ASSAYS

3.2.1 Introduction

To study the *in vitro* effects of the putative factor IX promoter, regions of the promoter can be linked to reporter genes and transfected into an appropriate eukaryotic cell line. Reporter genes such as the chloramphenicol acetyltransferase gene (CAT), β-glucuronidase (GUS) and the luciferase gene have been utilised widely in eukaryotic systems serving as indicators of transcriptional efficiency based on their expression levels. The reporter genes are located within a reporter gene expression vector appropriate for the cell lineage in which the reporter gene is expressed. Their

expression is facilitated through a promoter region which is fused upstream of the reporter gene allowing the transcripts initiated at the promoter to proceed through the reporter gene. Transcription from the promoter directs the enzymatic release of a protein specified by the coding region of the reporter gene *i.e* the CAT gene encodes the enzyme chloramphenicol acetyltransferase. The amount of enzyme released can be easily measured by functional assays.

The luciferase reporter gene was used in these studies to determine the *in vitro* expression from the factor IX promoter. The luciferase assay was chosen above the CAT-assay system due to its high sensitivity and reproducible nature. The minimal factor IX promoter (region -220 to +45) was cloned adjacent to the coding sequence for the luciferase gene within one of two expression vectors utilised in this study. Therefore when a eukaryotic cell line is transfected with the reporter gene construct, transcription initiated at the promoter would proceed through the luciferase reporter gene and the resulting transcripts translated into luciferase enzyme via the translational machinery of the host cell (Watson *et al.*, 1992). The luciferase gene which originates from the firefly *Photinus pyralis* encodes a 62 kDa luciferase polypeptide that catalyses the oxidation of beetle luciferin in a reaction requiring oxygen and ATP. This oxidation results in the production of a photon in a chemiluminescent reaction that is characterised by a yellow light. The amount of light emitted from the reaction is directly proportional to the transcriptional activity of the promoter. To assay the luciferase activity in the cells, a cell lysate is prepared and collected. Each lysate sample can then be measured for luciferase activity using a luminometer. Due to the sensitive nature and reproducibility of luciferase, the low levels of transcription produced from the minimal promoter can be accurately monitored. The transcriptional level measured from a reporter gene is, however, dependent on the efficiency of transfection obtained within the target cells *i.e* only a certain percentage of the eukaryotic cells will take up and express the DNA. The inherent variability that occurs with transfection efficiencies can be controlled by the addition of an internal control, which is incorporated into the transfection along with the experimental plasmids. Such an internal control should not affect the overall expression of the experimental plasmids but instead serves as an indicator of the amount of DNA taken up by the cells.

In these studies, a series of reporter gene constructs were prepared containing various mutations within the factor IX promoter region (-220 to +45). Each construct was assayed for transcriptional activity in the mammalian hepatoma cell line, Alexander.

3.2.2 Assembly of the Reporter Gene Construct

3.2.2.1 Preparation of the vector

Two different reporter gene expression vectors were used for the assembly of the reporter gene constructs. These were the pGL2 Enhancer vector, a 5.8 kb plasmid containing a SV40 enhancer but lacking any promoter sequence and pGL2 Basic, a 5.6 kb plasmid that lacks both promoter and enhancer sequences (Appendix 3). Both expression vectors contain the coding region of the luciferase gene, which is located 3' to a polylinker region. The expression vectors were digested in a 50 µl reaction with restriction enzymes *Xho* 1 and *Sst* 1 in order to linearise the vector DNA in preparation for the insertion of the promoter region. After digestion, the 5' phosphate groups of the vector DNA were removed prior to the concentration, purification and quantitation of vector DNA.

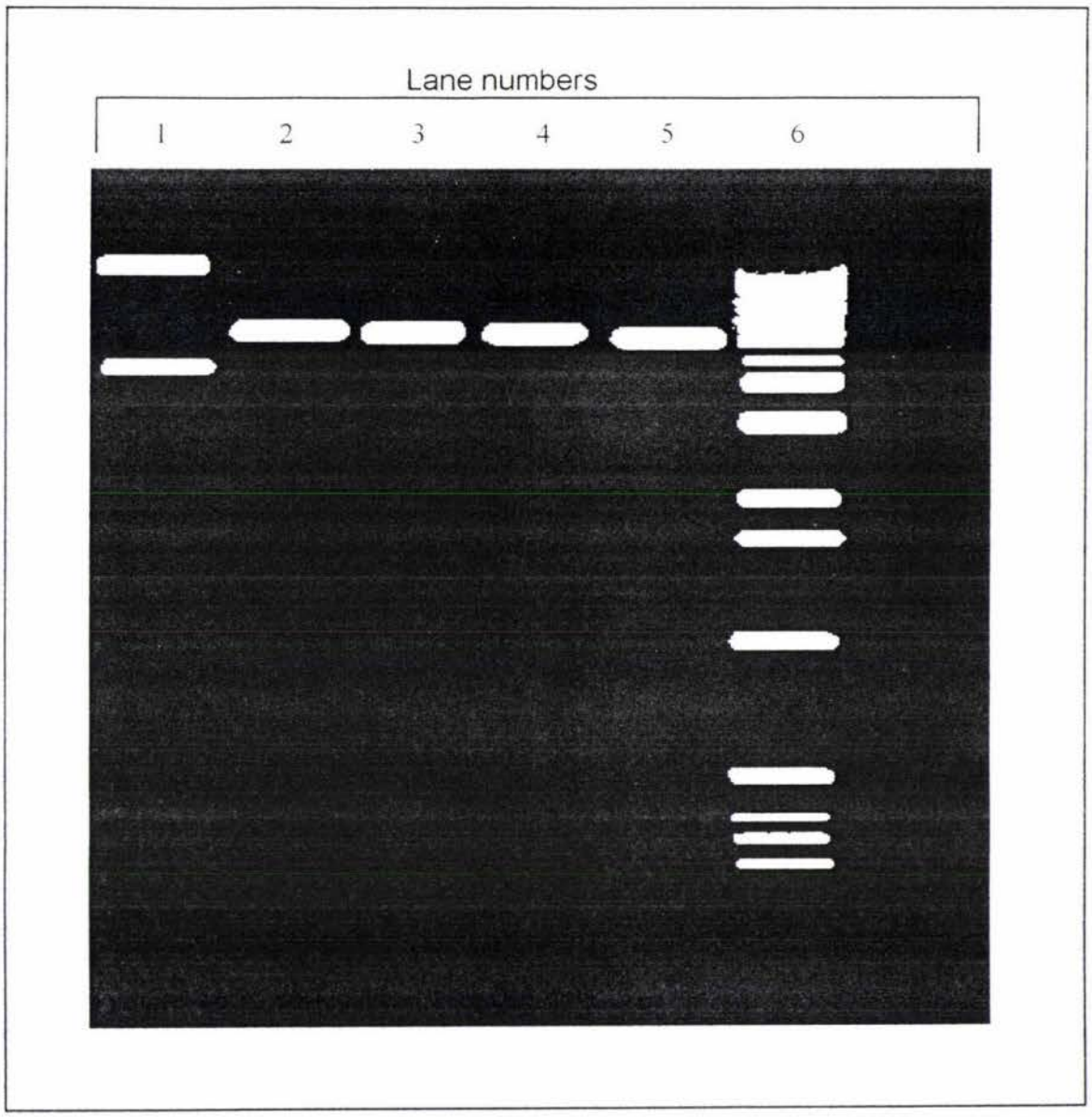


Figure 7. Digestion of the promoterless vector, pGL2 Basic with the restriction enzymes *Xho* 1 and *Sst* 1. A 5 µl aliquot from a 50 µl digestion reaction was analysed by electrophoresis in a 1 % agarose gel containing 1 x TAE. Two µl of ethidium bromide (10mg/ml) were incorporated into the gel in order for the bands to be visualised under a UV transilluminator.

1: undigested vector, pGL2 Basic

2, 3, 4 and 5: pGL2 Basic digested with restriction enzymes *Xho 1* and *Sst 1*.

6: 1 kb ladder.

3.2.2.2 Preparation of the factor IX promoter region

A 275 bp region of the factor IX promoter was amplified from the minigene construct pTG3954 (Appendix 1) in a PCR reaction using the high fidelity enzyme, *Pfu* polymerase (Fig. 8). The vector pTG3954 contains a 19 kb region of the factor IX gene which includes a 5 kb 5' flanking region. Mutations within the factor IX promoter were created using oligonucleotide-directed PCR mutagenesis (Appendix 2). The mutations disrupted the recognition sites of various transcription factors that interact with the factor IX promoter (Table 1). The amplified promoter inserts were subsequently digested with *Xho* 1 and *Sst* 1 restriction enzymes. *Xho* 1 is responsible for removing 5 bp from the 5' end of the factor IX promoter and *Sst* 1 digests 5 bp from the 3' end. This resulted in a 265 bp fragment corresponding to the -220 to +45 region of the factor IX promoter. Following digestion, the prepared promoter inserts were incorporated into the linearised vector DNA under standard ligation conditions. The correct insertion of the factor IX promoter region into the polylinker region of the expression vectors would enable transcription to occur from the luciferase gene when expressed in the Alexander cell line. The recombinant plasmids were then introduced into *E.coli* XL-blue cells via the $CaCl_2$ plasmid transformation method. Positive colonies established on LB-ampicillin plates were identified by restriction digestion with *Xho* 1 and *Sst* 1. Digestion of the recombinant plasmid released the 265 bp factor IX promoter insert from the vector DNA (Fig. 9). One of several positive colonies was selected to be sequenced in both directions to ensure that no abnormalities in the DNA had occurred during manipulation. After the correct sequence had been obtained, the recombinant plasmid was grown up on a large scale for use in reporter gene construct assays.

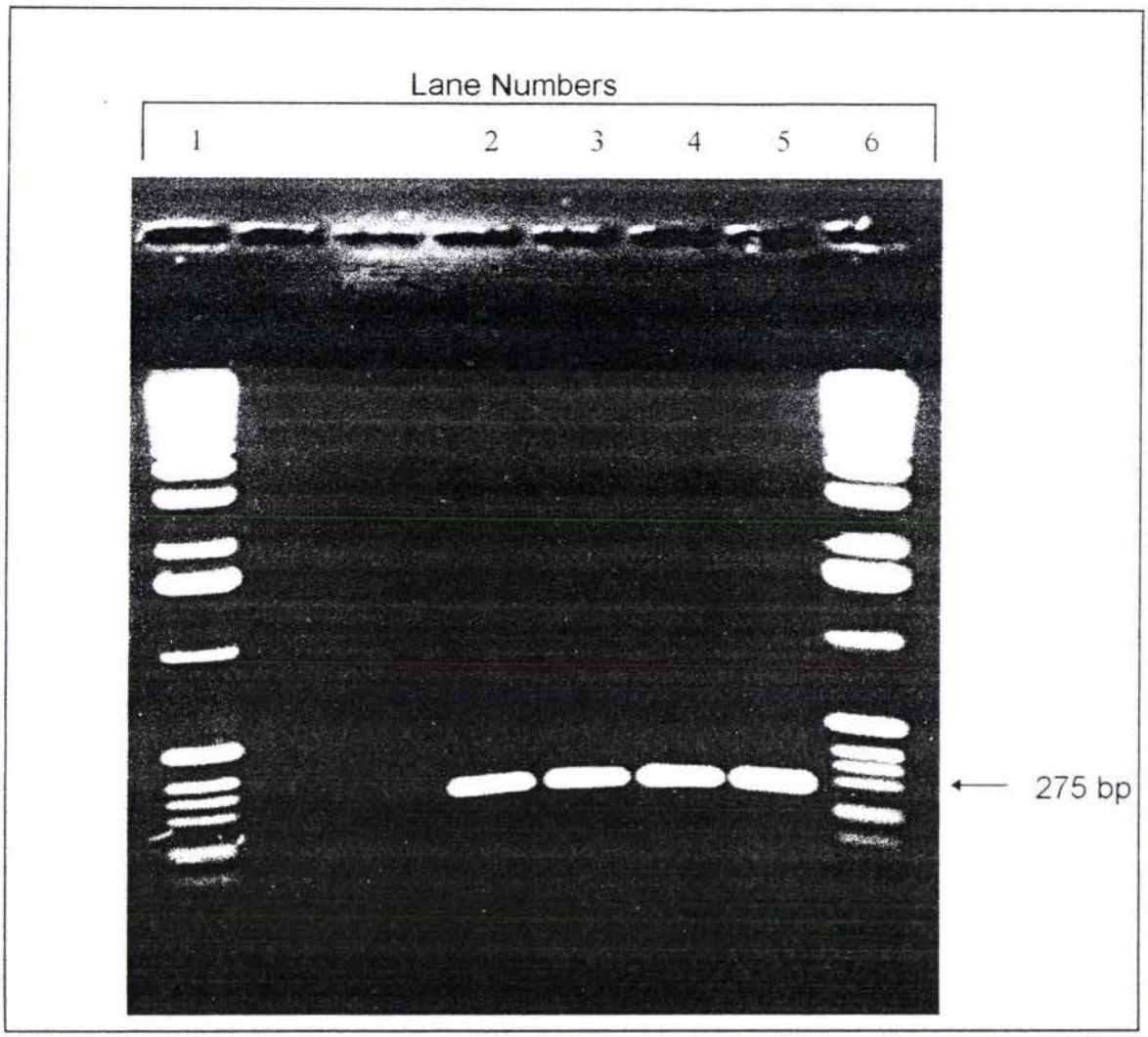


Figure 8. Amplification of the pTG3954 template by PCR mutagenesis. A 5 μl aliquot of each PCR reaction was analysed by electrophoresis in a 1 % agarose gel [2 μl of EtBr (10 mg/ml)] containing 1 x TAE. The 275 bp fragment was amplified using the high fidelity enzyme, *Pfu* polymerase in conjunction with oligonucleotides to create mutations in the binding sites for various transcription factors.

1: 1kb ladder (BRL)

2: pTG3954 template and an oligonucleotide primer containing a mutation in the putative HNF4 site (region +20 to +45)

3: pTG3954 template and an oligonucleotide primer containing a mutation in the C/EBP/DBP site (region –220 to –202)

4: pTG3954 template and an oligonucleotide primer containing a double mutation in the putative HNF4 site (region +20 to +45) and the C/EBP/DBP site (region –220 to –202).

5: male genomic DNA with a +13 A to G mutation

6: 1 kb ladder (BRL)

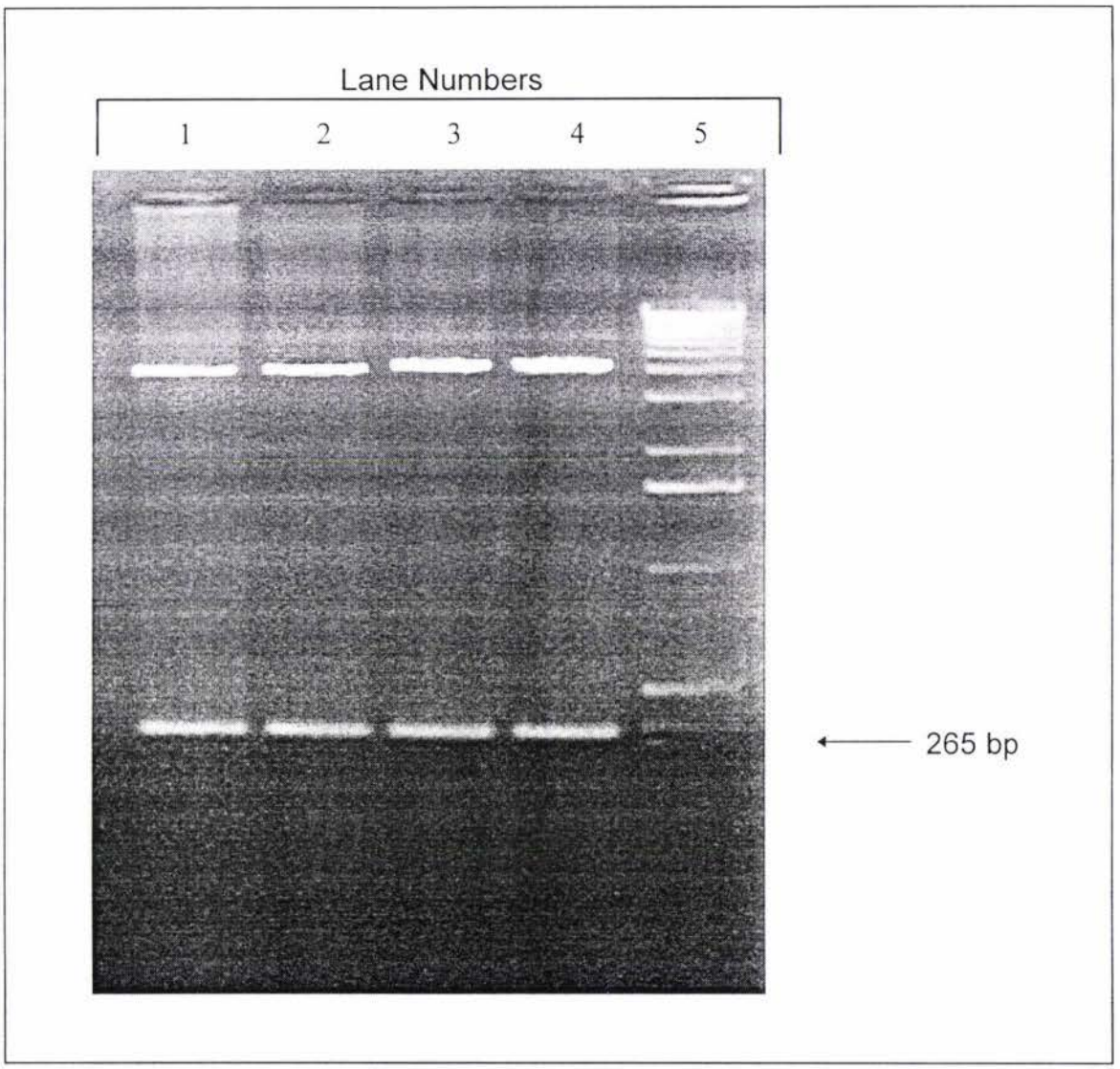


Figure 9. Analysis of factor IX promoter inserts from the expression plasmid, pGL2 Basic. The 265 bp promoter insert was separated from the pGL2 Basic vector by restriction digestion with *Xho* 1 and *Sst* 1. Each digested sample was analysed on a 1 % agarose gel [2 μl of EtBr (10 mg/ml)] in 1 x TAE.

1: pGL2 Basic/ promoter insert –220 to +45 with a mutation in the putative HNF4 site at region +20 and +45

2: pGL2 Basic/ promoter insert –220 to +45 with a mutation in the C/EBP/DBP site at region -220 to -202

3: pGL2 Basic/ promoter insert –220 to +45 with a mutation in both the HNF4 and C/EBP/DBP sites

4: pGL2 Basic/ promoter insert –220 to +45 with a mutation in the +13 A to G site.

5: 1 kb ladder (BRL).

Table 1. Oligonucleotides used for PCR mutagenesis.

Oligonucleotides	Nucleotide change	Comments
La-1/3137	-202 A to C	Disrupts the C/EBP/DBP binding site at region -220 to -202
La-2/3136	+26 G to C	Disrupts the putative HNF4 binding site at region +20 to +45
La-1/3137 and La-2/3136	-214 A to C; +24 A to C	Disrupts both the C/EBP/DBP site at region - 220 to –202 and the HNF4 site at region +20 to +45
	+13 A to G	Disrupts a C/EBP site at region +1 to +18
La-1/3137	+13 A to G; removed thymine residue removed adenine residue	Disrupts both a C/EBP/DBP site at region - 220 to –202 and a C/EBP site at region +1 to +18

3.3 POTENTIAL USE OF THE REPORTER GENE CONSTRUCTS

Mutational analysis of the factor IX promoter was undertaken in the current studies to help determine the importance of transcription factors involved in the regulation of the factor IX promoter. To date, such analysis has been restricted to mutations that have already been identified within the haemophiliac population.

Reporter gene constructs containing mutations at each end of the factor IX promoter (region –220 to +45) were created in order to analyse the effects of these mutations on transcriptional activity (Fig. 10). This involved a time-consuming procedure of sequential steps which were occasionally repeated because of unforeseen

and unwanted mutations occurring during genetic manipulation. Once prepared, the reporter gene constructs could not be utilised for *in vitro* studies within the Alexander cell line because of financial constraints.

The mutations created were designed to examine the role of the CCAAT/enhancer binding protein (C/EBP), the D-site binding protein (DBP) and hepatic nuclear factor 4 (HNF4) protein, in the transcriptional regulation of the factor IX promoter by disrupting their putative binding sites. The extent to which these mutations affected promoter expression would have subsequently been compared to the wild-type promoter (region –220 to +45) as well as the Leyden mutations that occur within the factor IX promoter, such as the -6 G to C, -20 T to A and the Brandenburg -26 G to C mutation.

The importance of the proximal C/EBP/DBP (region -220 to -202) and HNF4 (region +20 to +45) binding sites in the transcriptional regulation of the factor IX promoter are not well understood. A synergistic interaction between C/EBP and DBP at -220 to -202 of the factor IX promoter has been implicated in the post-pubertal recovery of haemophilia B by several research groups (Picketts *et al.*, 1993). Previous *in vitro* studies carried out by Picketts *et al.* (1993) demonstrated that the transcriptional disruption created by a mutation at -5 could be compensated for by the combined interaction of C/EBP and DBP at region -220 to -202 of the factor IX promoter. In similar experiments, Franklin (1995) proposed that the observed increase in factor IX transcription from promoter constructs (region -189 to +20) containing either a -5 A to T or -6 G to A mutation was the result of truncating the C/EBP/DBP region. The subsequent exposure of the mutated promoter region to an alternative set of transcription factors that recognised and bound to the new mutated sequence was proposed to result in the observed increase in transcription (Franklin, 1995).

Analysis of the C/EBP/DBP binding site (region -220 to -202) by research groups has been based primarily on reporter gene assays that exclude this region from factor IX promoter constructs. Naturally occurring mutations within this site have not been reported in the population; the role of C/EBP/DBP in the regulation of the factor IX transcription was to be examined by creating a novel mutation within this region

(Fig. 10A). *In vitro* mutagenesis would provide an opportunity to analyse the importance and subsequent involvement of the two transcription factors, C/EBP and DBP, in normal factor IX gene expression. The extent of transcriptional disruption created by this mutation could then be compared within the Alexander cell line to the wild-type promoter (region -220 to +45) as well as to the Leyden mutations such as -6 G to C, -20 T to A, and the Brandenburg -26 G to C mutation. This would enable a comparison to be made between the type of transcription factor binding site which has been disrupted and the extent of the transcriptional disruption.

The incorporation of the expression plasmids for the C/EBP and DBP transcription factors into transient transfections would also enable a direct comparison to be made between the exclusion of the C/EBP/DBP site and the ability of the factor IX promoter to enhance transcription in the presence of C/EBP and DBP.

The nucleotide sequence at region +20 to +45 of the factor IX promoter has been postulated to be a putative HNF4 binding site owing to the partial sequence homology of this region to the consensus DNA binding site for HNF4 at region -15 to -30 (Pang *et al.*, 1990). Little is known about the putative HNF4 binding site and its possible involvement in the transcriptional regulation of the factor IX promoter. *In vitro* analysis within this region has been limited, thus providing a novel opportunity to examine the putative role of this site in normal factor IX expression using *in vitro* mutagenesis. A second construct was therefore created containing a mutation within the putative HNF4 site of the factor IX promoter (Fig. 10B). This construct was used in transient transfections in the Alexander cell line (Chapter 3.3.8) to analyse the effects of the disruption of this site on transcriptional activity.

In retrospect, two other mutant constructs could have been prepared so that the binding of the HNF4 transcription factor to the factor IX promoter could be examined. This would have involved mutating the consensus binding site for HNF4 at region -15 to -30 as well as mutating both HNF4 sites at -15 to -30 and +20 to +45. The extent of transcriptional disruption created from mutating each HNF4 site respectively and mutating all HNF4 sites, on the expression of the factor IX promoter could then be analysed within the Alexander cell line. This would help elucidate the importance of

HNF4 on factor IX transcription, and the specificity of HNF4 for each of the two consensus sites.

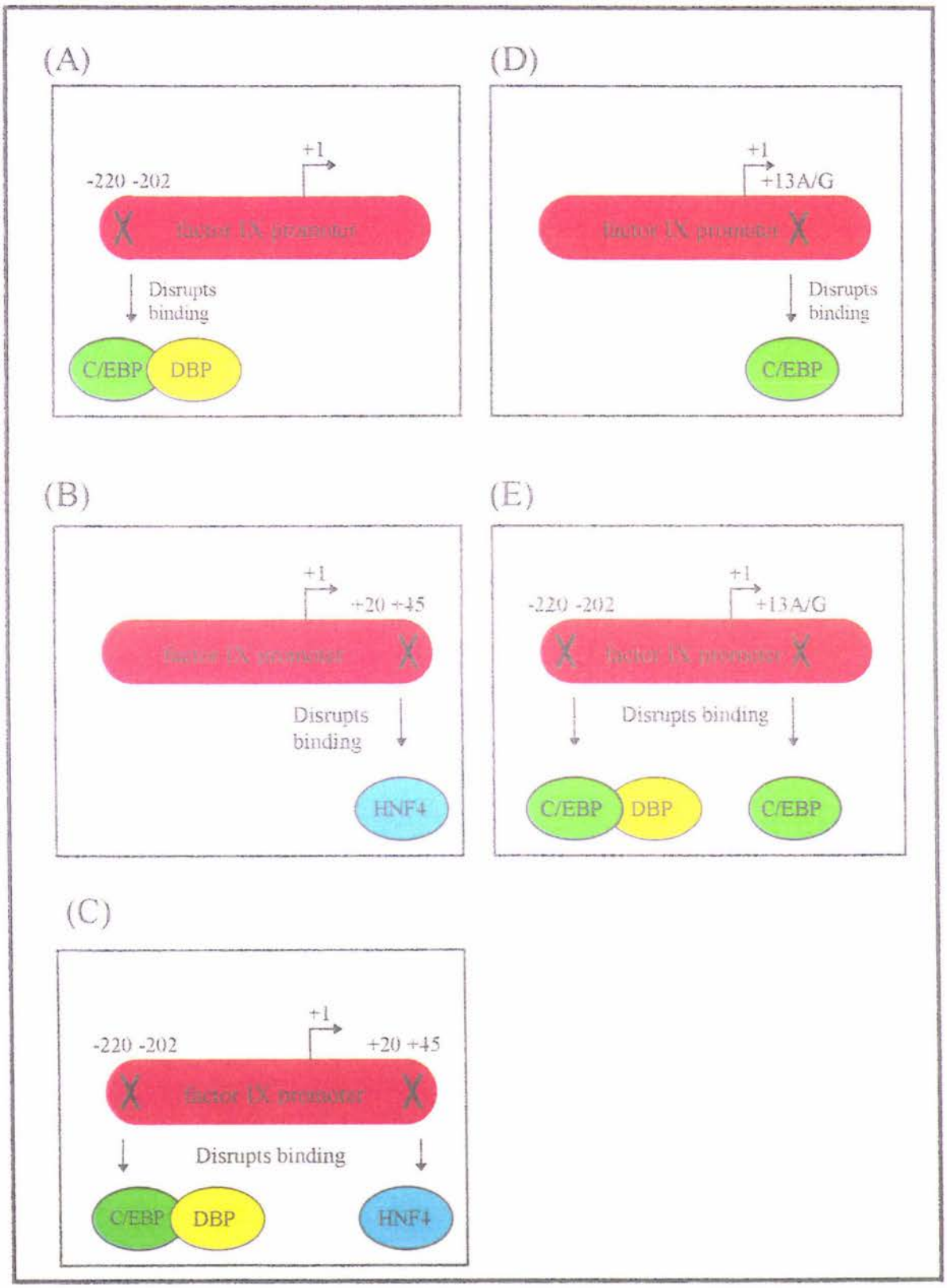


Figure 10. A schematic representation of the mutations created within the human factor IX promoter.

A third reporter gene construct was created containing a double mutation within the proximal C/EBP/DBP (region -220 to -202) and putative HNF4 binding (region +20 to +45) sites (Fig. 10C). This construct was prepared for the purpose of analysing the extent of transcriptional disruption created by disabling both proximal sites of the factor IX promoter. The extent of transcription disruption could be compared to both wild-type and Leyden mutant promoters. The incorporation of reporter gene constructs containing mutations within either the proximal C/EBP/DBP site (region -220 to -202), or the putative HNF4 site (+20 to +45) in transient transfections, would assist in determining the importance of the C/EBP/DBP and HNF4 binding sites in the transcriptional activation of the factor IX promoter. Additional co-transfection experiments using the expression plasmids for the transcription factors C/EBP and DBP, as well as the expression plasmid for HNF4 with normal and mutant promoter constructs would enable a study of the potential enhancement of the factor IX promoter. This would also assist in elucidating the roles of each transcription factor in the regulation of the factor IX promoter

A mutation within the +13 region of the factor IX promoter has been associated with a severe form of Haemophilia B Leyden (Giannelli *et al.*, 1994). The clotting levels within patients suffering from this disorder are less than 1 % of normal activities prior to puberty but gradually increase to ~30 % of normal after the onset of puberty (Giannelli *et al.*, 1994). The severity of this mutation is created by the disruption of a C/EBP binding site at nucleotides +1 to +18 (Crossley *et al.*, 1990). Previous *in vitro* studies have produced contradictory reports on the extent of transcriptional disruption created by this mutation. Crossley and Brownley (1990) showed that promoter (-189 to +21) constructs containing the +13A to G mutation did not impair promoter activity within the HepG2 cell line. In contrast, Reijnen *et al.* (1994) reported a 2.5 fold reduction in transcriptional activity with the +13 A to G mutant promoter (-192 to +38) construct. The difference between these studies was assumed to be the result of the differential expression of endogenous C/EBP within the HepG2 cell line (Reijnen *et al.*, 1994). However, it is possible that the exclusion of the proximal C/EBP/DBP site at region -220 to -202 of the factor IX promoter in both reports may have altered protein interactions. In order to clarify these experiments within the Alexander cell line, a fourth reporter gene construct was prepared, containing a +13 A to G mutation within

the factor IX promoter (region -220 to +45) (Fig. 10D). The extent of transcriptional disruption created by this mutation could therefore be analysed by transient transfections. The extent to which the promoter could also be transactivated by the co-transfection of the expression plasmid for C/EBP would also help in determining the importance of the region in factor IX expression.

In addition to the interaction of the C/EBP transcription factor with the +1 to +18 region of the factor IX promoter, the role of DBP at this site was to be analysed as well. Several reports have confirmed the existence of a DBP binding site overlapping the C/EBP consensus sequence at region +1 to +18 (Reijnen *et al.*, 1994). The interaction of DBP with the factor IX promoter at this site is not well understood. Interestingly, a mutation within the +13 region of the factor IX promoter was found to disrupt the C/EBP site only without interfering with the binding of the DBP protein (Reijnen *et al.*, 1994). Therefore the aim was to examine the possible binding and transactivation of the factor IX promoter containing a +13 A to G mutation in the presence of the expression plasmid for DBP.

The last reporter gene construct that was created involved a double mutation within the factor IX promoter, disrupting both C/EBP binding sites at region +1 to +18 and region -220 to -202 (Fig. 10E). This construct was to be utilised in transient transfections with the previous reporter construct containing a mutation within the C/EBP site at +1 to +18. This study would enable a comparison to be made between each of the C/EBP sites and the resultant transcriptional disruption of the factor IX promoter. In turn, this would help elucidate the importance of the C/EBP transcription factor in the regulation of the factor IX promoter and determine which of the two C/EBP sites had a greater effect on transcription. Co-transfection of these reporter gene assays with expression vectors for C/EBP and DBP could also be examined in order to see if these transcription factors could bind and enhance the promoter when mutated.

Table 2. A summary of the reporter gene constructs that were prepared or utilised in these studies

Reporter constructs	Comments
pGL2B -6 G/A	Disrupts the unidentified transcription factor at region +5 to +15
pGL2B -6 G/C	Disrupts the unidentified transcription f actor at region +5 to +15
pGL2B -20 T/A	Disrupts the HNF4 binding site at region -15 to -30
pGL2B -26 G/C	Disrupts the ARE element (-36 to -21) and the neighbouring HNF4 site (-15 to -30)
pGL2B Δ +20 to +45	Disrupts the putative HNF4 site (+20 to +45)
pGL2B +13 A/G	Disrupts the C/EBP binding site (-15 to -30)
pGL2B +13 A/G Δ -220 to -202	Disrupts the both C/EBP binding sites (-15 to -30 and -220 to -202)
pGL2B Δ -220 to 202	Disrupts the C/EBP/DBP binding site at region -220 to -202
pGL2B Δ -220 to -202 /+20 to +45	Disrupts the C/EBP/DBP site (-220 to -202) and the putative HNF4 site (+20 to +45)

Mutational analysis provides a source of information on gene expression and the transcriptional factors involved. Future studies using these reporter gene constructs may therefore lead to a greater understanding of the molecular mechanism involved in the regulation of the factor IX gene.

3.4 OPTIMISATION OF THE REPORTER GENE ASSAY SYSTEM

In order for optimal levels of luciferase transcription to be obtained with the reporter gene constructs and the Alexander cell line, experiments were initially carried out to test the different variables that may influence luciferase expression. The cell densities used to seed each transfection experiment were important to reporter gene construct expression as they affected the success of each transfection experiment by the cells ability to take up and express DNA. The two different sized tissue culture plates utilised during the course of this study supported different cell densities. Experiments were carried out to determine the amount of cells required for optimal luciferase activity when using the two tissue culture plates. Alexander cells that had been seeded into tissue culture plates at different densities were transfected with the control plasmid, pGL2 Control, which contains the luciferase reporter gene under the control of the SV40 promoter sequence, or a promoterless vector pGL2 Basic. The cells had been initially passaged (80 % confluent) from a T80 flask and resuspended into 3 ml of complete MEM. The resuspended cells were then dispensed dropwise from a 5 ml plastic pipette into each tissue culture plate (containing complete MEM) in preparation for transfection. The larger 6 cm tissue culture plates required 3 drops (0.6 ml) (Fig. 11) of cells from a 3 ml resuspension whereas the smaller multiwell plates only required 1 drop (0.2 ml) (Fig. 12) of cells for maximum luciferase activities. Overly dense cells used for transfection affected the cell viability and thus effected the transfection efficiencies.

The calcium phosphate precipitation method of transfection was found to be most efficient when the DNA precipitate was left on the cells for 16 to 24 hours. Any shorter or longer period of time was found to decrease the overall efficiency of the transient transfection. The amount of plasmid DNA required to produce optimal luciferase expression differed with the different sized transfection plates used in the course of this study. The larger 6 cm plates required 10 μg of reporter construct DNA and 5 μg of β-galactosidase expression vector, pCH110, per transfection. The smaller multiwell tissue culture plates required only one fifth of the amount of DNA used for the larger plates to produce optimal transfection levels. The amount of plasmid DNA required to produce optimal luciferase expression had been already determined in previous studies carried out by Franklin (1995).

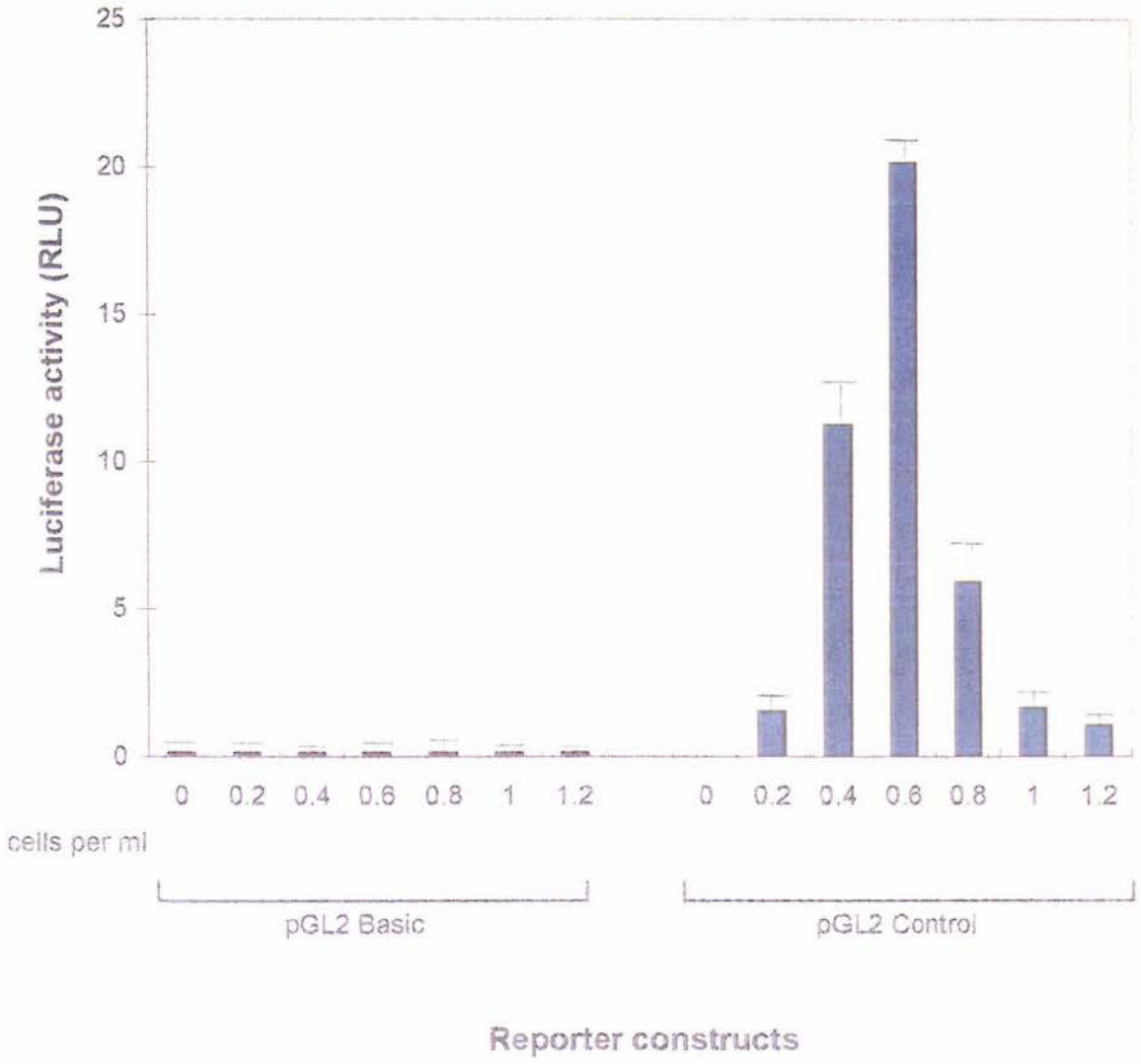


Figure 11. Optimisation of cell density in transient transfections. A determination of the cell density required for optimal transient transfections using the 6 cm tissue culture plates. The Alexander cells were seeded into the tissue culture plates at different densities (ml) the day before transfections were carried out. For each experiment, the Alexander cell line was transfected with either 10 μg of the control vector, pGL2 Control, or 10 μg of the promoterless vector, pGL2 Basic. In addition, 5 μg of the β-galactosidase encoding plasmid pCH110 was added as an internal transfection control. The luciferase activity (measured in relative light units) given for each construct was the average of three independent experiments, each containing three replicates.

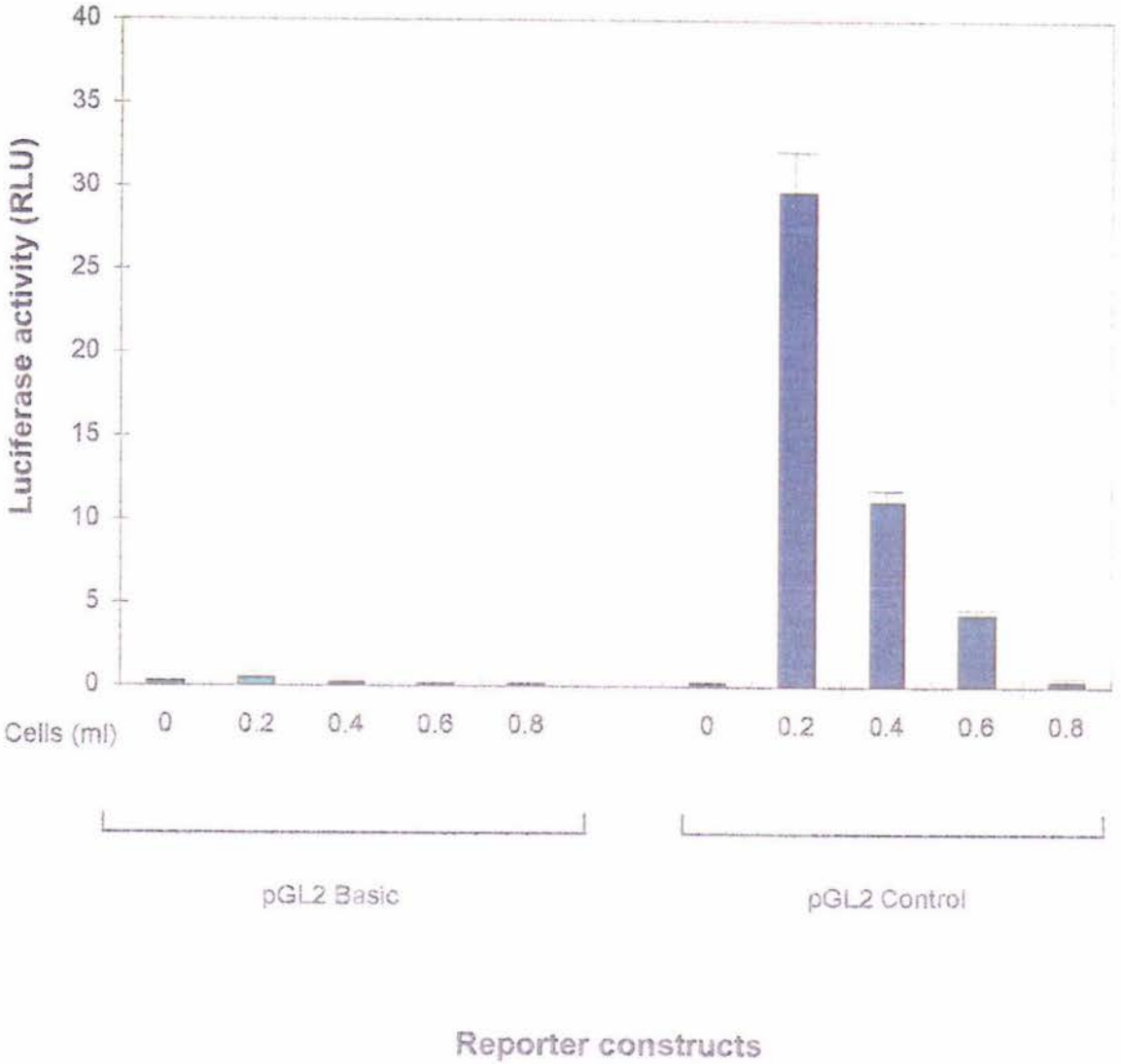


Figure 12. Optimisation of cell density in transient transfections. A determination of the cell density required for optimal transient transfections using the 15 mm tissue culture plates. The Alexander cells were seeded into the tissue culture plates at different densities (ml) the day before transfections were carried out. For each experiment, the Alexander cell line was transfected with either 2 μg of the control vector, pGL2 Control, or 2 μg of the promoterless vector, pGL2 Basic. In addition, 1 μg of the β-galactosidase encoding plasmid pCH110 was added as an internal transfection control. The luciferase activity (measured in relative light units) given for each construct was the average of three independent experiments, each containing three replicates.

3.5 ANALYSIS OF THE -6 NUCLEOTIDE REGION

3.5.1 Introduction

Patients carrying a rare G to C transition at position -6 (relative to the +1 transcriptional start site; Anson *et al.*, 1984) suffer from a severe form of haemophilia B. This mutation results in severe bleeding tendencies in patients with pre-pubescent blood clotting activities less than 1 % of normal clotting activities (Giannelli *et al.*, 1994; Viduad *et al* ., 1993). The G to C transition at -6 eliminates a normal *Taq* 1 restriction site present within the wild-type sequence (Viduad *et al.*, 1993) and can therefore serve as a rapid screening method for potential carriers and patients (Viduad *et al.*, 1993). Like most other Leyden-specific mutations, the clinical symptoms resulting from the -6 G to C mutation ameliorate after puberty with blood clotting activities reaching ~30 % of normal (Giannelli *et al.*, 1994).

The severity of haemophilia B fluctuates with the different nucleotide substitutions at the -6 region of the promoter (Franklin, 1995). For example, the -6 G to A mutation, which involves a CpG dinucleotide (Green *et al.*, 1990) results in a mild form of haemophilia B. Patients suffering from the -6 G to A mutation have an initial blood clotting activity of 13 % (Giannelli *et al.*, 1994) prior to puberty, which subsequently increases to 70 % after puberty. *In vitro* analysis of the -6 region of the factor IX promoter and its involvement in the regulation of the factor IX gene is currently limited. Previous studies using the hepatoma cell line, HepG2, have shown that both the -6 G to A and -6 G to C mutation downregulate the factor IX promoter to different extents - the latter of which was shown to have a more deleterious effect on promoter activity (Hirosawa *et al.*, 1990). Although both of the mutations in the –6 region probably disrupt the binding of the same transcription factor (region +5 to -15), which has yet to be identified, the clinical data available suggests that the two mutations result in different blood clotting activities both prior to and post-puberty (Giannelli *et al.*, 1994). Although certain discrepancies lie in the way the clinical data was collated *i.e* variation between factor IX clotting activities in patients suffering from the same mutation and variation in the patients age (Giannelli *et al.*, 1994), the overall data presented by the various laboratories suggests that the different nucleotide substitutions

in the -6 region of the factor IX promoter have different effects on factor IX transcription and thus factor IX expression. The difference in phenotypic severity between the two mutations therefore suggests an importance of the -6 region in factor IX gene expression (Viduad *et al.*, 1993).

Reporter gene assays were performed to analyse the extent to which the -6 G to C mutations downregulate the factor IX promoter (region -220 to +45) in comparison to the wild-type sequence. The effect on transcription from the factor IX promoter of four mutations at -5 and -6 has previously been analysed by Franklin (1995). This study used two different reporter gene constructs containing the -189 to +20 and the -220 to +45 region of the factor IX promoter. The experimental system developed by Franklin (1995) was used in the current study to determine the effects of mutations in the -220 to -202 and +20 to +45 region of the factor IX promoter.

Preliminary experiments were carried out in order to ascertain that the Alexander cell line could be used as a suitable system in which to study the effect of both HNF4 and C/EBP on transcription.

3.5.2 Results.

Alexander cells were transiently transfected with either the normal or mutant (-6 G to C and -6 G to A) reporter gene constructs. Cell lysates were prepared and assayed for luciferase gene activity. All transfections were carried out using the 6 cm sized tissue culture plates. The results are summarised in Fig. 13 and the data presented in Table 3.

Table 3. Luciferase activity in Alexander cells comparing normal and Leyden promoters. Each construct contained the region -220 to +45 of the factor IX promoter fused to the luciferase gene in the promoterless vector pGL2 Basic. The standard deviation of four independent experiments is shown.

Promoter Construct	Relative Luciferase Activity (%)
Normal	100 ± 16.5
Leyden (-6 G to C)	41.1 ± 6.8
Leyden (-6 G to A)	78.3 ± 4.1
pGL2 Basic	12.6 ± 2.3

All reporter gene assays are expressed as a 'relative luciferase activity' whereby the amount of luciferase is shown as a percentage relative to the wild-type promoter, which was arbitrarily set at 100. Results were averaged from four separate experiments, each of which contained triplicate transfections of all reporter constructs.

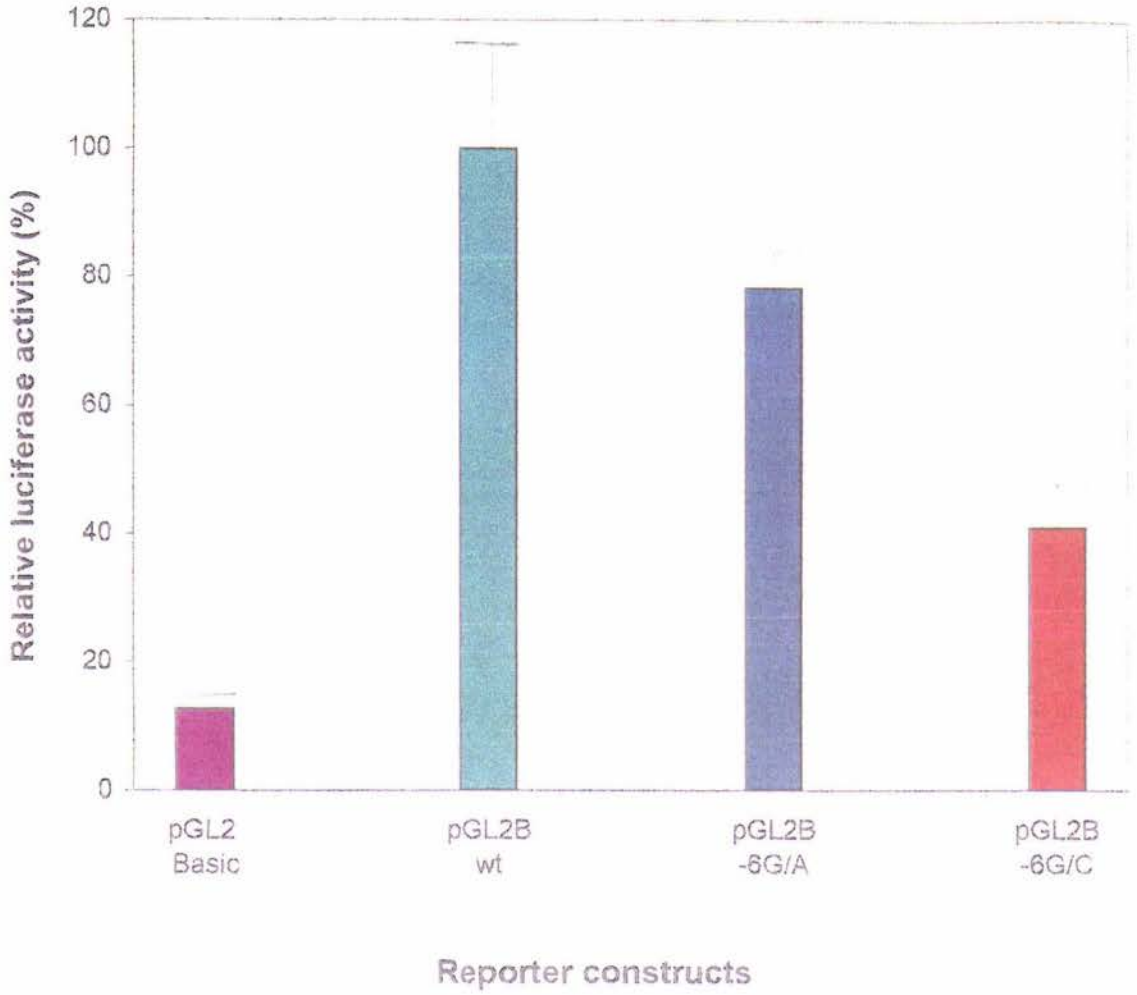


Figure 13. A comparison of the -6 G to A and -6 G to C promoter (region -220 to +45) mutations. The promoterless vector, pGL2 Basic, was used as a control and the β-galactosidase encoding plasmid, pCH110 was used as a transfection control. The amount of luciferase is shown as a percentage relative to the plasmid pGL2B wt, which is arbitrarily set at 100. All results are compiled from at least four independent experiments, each containing three replicates of each plasmid. The standard deviation of the mean is shown for each construct. Extracts were prepared 48 hours after transfection and the luciferase and β-galactosidase activities measured.

3.5.3 Discussion.

The data presented in Fig. 13 supported reports by both Reijnen *et al.* (1994) and Franklin (1995). Functional studies performed in Alexander cells showed that both mutations in the -6 region downregulate the factor IX promoter (region -220 to +45) to different extents when compared to the normal promoter construct. The -6 G to C mutation had a more inhibitory effect on promoter activity with luciferase activities of only 41.1 % of the normal promoter activity. This 2.4 fold reduction in promoter activity was similar to the reduction observed in studies reported by Reijnen *et al.* (1994), using the CAT-expression system. In their studies, the factor IX promoter (region -192 to +38) constructs containing the -6 G to C mutation decreased the promoter activity ~3 fold when expressed in the HepG2 cell line. The slight discrepancies in the transcriptional activation between the two studies may have been a result of the different cell lines used in each study or the different promoter regions that were incorporated into the reporter gene constructs. As a consequence, the shorter promoter region utilised by Reijnen *et al.* (1994) may have altered protein-protein and protein-DNA interactions due to the exclusion of various transcription factor recognition sites.

The extent of transcriptional disruption created from the -6 G to C mutation in these assays was not as extensive as the affects created *in vivo*. For example, the blood clotting activities from patients suffering from the -6 G to C mutation are less than 1 % of the normal blood clotting activities. Although the levels of factor IX in the plasma cannot be directly correlated to the levels of luciferase produced from an *in vitro* system, as not all elements may be tested in this system, the *in vitro* system provides a means of examining the relative effect of the mutations on promoter activity (Picketts *et al.*, 1994).

The reporter gene assays for the -6 G to A mutation indicated a less severe effect on promoter inhibition than the effects created by the -6 G to C mutation. Figure 13 shows that the -6 G to A mutation reduced factor IX promoter (region -220 to +45) activity to 78.3 %. This result was consistent with promoter (region -220 to +45) activities reported by Franklin (1995) using the luciferase assay system in the Alexander

cell line. In contrast, transient transfections performed by Hirosawa *et al.* (1990) using the CAT-expression system in HepG2 cells, demonstrated that the -6 G to A mutation downregulated the factor IX promoter (region -419 to +29) to ~38.5% of normal promoter activity. The marked reduction in promoter activity observed by Hirosawa *et al.* (1990) with the -6 G to A mutation may have been a direct result of the longer promoter insert incorporated into their CAT-reporter constructs. Elongation of the promoter region to such an extent may have introduced other transcriptional recognition binding sites or even repressor elements that may have effectively altered protein interactions and thus the overall expression from the factor IX promoter.

An even lower promoter expression was observed by Crossley and Brownlee (1990) when the -6 G to A mutation was expressed in HepG2 cells. These authors found that the -6 G to A mutation reduced promoter activity to background levels similar to those observed with their promoterless vector. The extensive reduction in promoter activity may be due in part to the shortened factor IX promoter region utilised in this study (region –189 to +21). This promoter region eliminated or disrupted the putative binding sites for the transcription factors HNF4, at region +20 to +45, and C/EBP/DBP, at region -219 to -202. The elimination of such binding sites for transcription factors involved in factor IX expression may effectively alter protein interactions with the promoter and subsequently alter promoter activity.

The mild phenotypic severity associated with the -6 G to A mutation may be the result of a less severe disruption of the binding site for the unidentified transcription factor in comparison to other Leyden mutations. Alternatively, the unidentified transcription factor may not play such an important role in factor IX gene expression as other transcription factors that interact with the promoter (Hirosawa *et al.*, 1990; Picketts *et al.*, 1994; Kurachi *et al.*, 1994). Therefore the disruption of the unidentified transcription factor binding site which may alter subsequent interactions between the transcription factor and promoter region, would not be as detrimental to overall factor IX gene expression. This could also explain why mutations at the -5 region of the promoter, which also disrupt the binding of the unidentified transcription factor, result in a mild form of Haemophilia B Leyden. The latter hypothesis however fails to explain why the -6 G to C mutation results in a more severe form of haemophilia B.

The variation in transcriptional activity of the factor IX promoter between the -6 G to A and -6 G to C mutation may be due to the availability of hydrogen binding sites that lie within the major groove of DNA. The major groove of DNA containing purines such as adenine and guanine may contain more hydrogen binding sites than a major groove consisting of pyrimidine residues (*i.e* cytosine and thymine) which contain fewer hydrogen binding sites (Pavlevtich and Pabo, 1991).

The hydrogen binding sites play an important role in protein-protein interactions. In particular, they are involved in directing the orientation of specific transcription factors when binding to the promoter DNA (Pavlevtich and Pabo, 1991). Therefore any alteration in the number of hydrogen binding sites may effectively alter the binding efficiency of a transcription factor to a promoter. This may in part, explain why the -6 G to C and -5 A to T mutations within the promoter have a more inhibitory effect on gene expression than -5 A to G and -6 G to A mutations (Giannelli *et al.*, 1994). The differences in pre- and post-pubertal blood clotting activities within patients suffering from the -6 G to C and -6 G to A mutation along with the possible role of the pyrimidine and purine bases suggests that the -6 region of the factor IX promoter may have an important role in the regulation of factor IX transcription.

The reporter gene assay results in Fig. 13 indicated that the experimental system within the Alexander cell line was capable of promoting transcription from reporter gene constructs and thus enabled further investigations of the factor IX promoter to be carried out within this cell line.

3.6 A COMPARISON OF LUCIFERASE EXPRESSION FROM THE pGL2 BASIC AND pGL2 ENHANCER VECTORS

3.6.1 Introduction

The use of an optimised reporter gene assay system to study the *in vitro* effects of promoter mutations on transcriptional initiation is critical for an accurate representation of promoter activity within a cultured cell line.

Initially two expression vectors were utilised in these studies to promote optimal expression of the luciferase gene. These were the pGL2 Basic and pGL2 Enhancer vectors. The pGL2 Basic vector is similar in structure to the pGL2 Enhancer vector, except the latter vector contains an SV40 enhancer region located upstream to the factor IX promoter insert. The enhancer element is thought to promote transcription from the luciferase gene so that small transcriptional changes that occur within the promoter can be efficiently detected by functional assays.

The Basic vector was initially used in transient transfections with the Alexander cell line. However, the overall expression from this construct was low. As a result preliminary studies using the pGL2 Enhancer vector were trialed. One might therefore expect to see an increase in luciferase activity and a significant difference between wild-type and mutant promoter constructs. If a higher level of expression is obtained with the pGL2 Enhancer vector, then the enhancer could be used for reporter gene construction instead of the pGL2 Basic vector.

Reporter gene assays were carried out to measure transcriptional activation from the factor IX promoter (region -220 to +45) inserted into both the pGL2 Basic and pGL2 Enhancer vectors. The different levels of luciferase activity attained with the -6 G to C promoter mutations (region -220 to +45) within the pGL2 Basic and pGL2 Enhancer vectors were also compared.

3.6.2 Results

Alexander cells were transiently transfected with either the pGL2 Basic or pGL2 Enhancer vectors that contained both the normal and mutant factor IX promoter (region -220 to +45) inserts. All transient transfections were carried out using the 6 cm sized tissue culture plates. Cell lysates were prepared and assayed for luciferase activity. The luciferase activities are expressed in relative light units to enable a direct comparison between the promoter activity of each reporter construct. The data for the results are presented in Table 4 and in Fig. 14.

Table 4. Luciferase activity in Alexander cells comparing normal and Leyden promoters. Each construct contained the region -220 to +45 of the factor IX promoter fused to the luciferase gene in the promoterless vector pGL2 Basic or pGL2 Enhancer. The standard deviation of the mean from three independent experiments is shown.

Promoter insert	Relative light units ± S.D	
	Vector	
	pGL2 Basic	pGL2 Enhancer
Normal	4.3 ± 1.1	0.4 ± 0.1
Mutant (-6 G to C)	1.3 ± 0.3	3.1 ± 1.3
Control (vector only)	0.3 ± 0. 1	0.2 ± 0.0

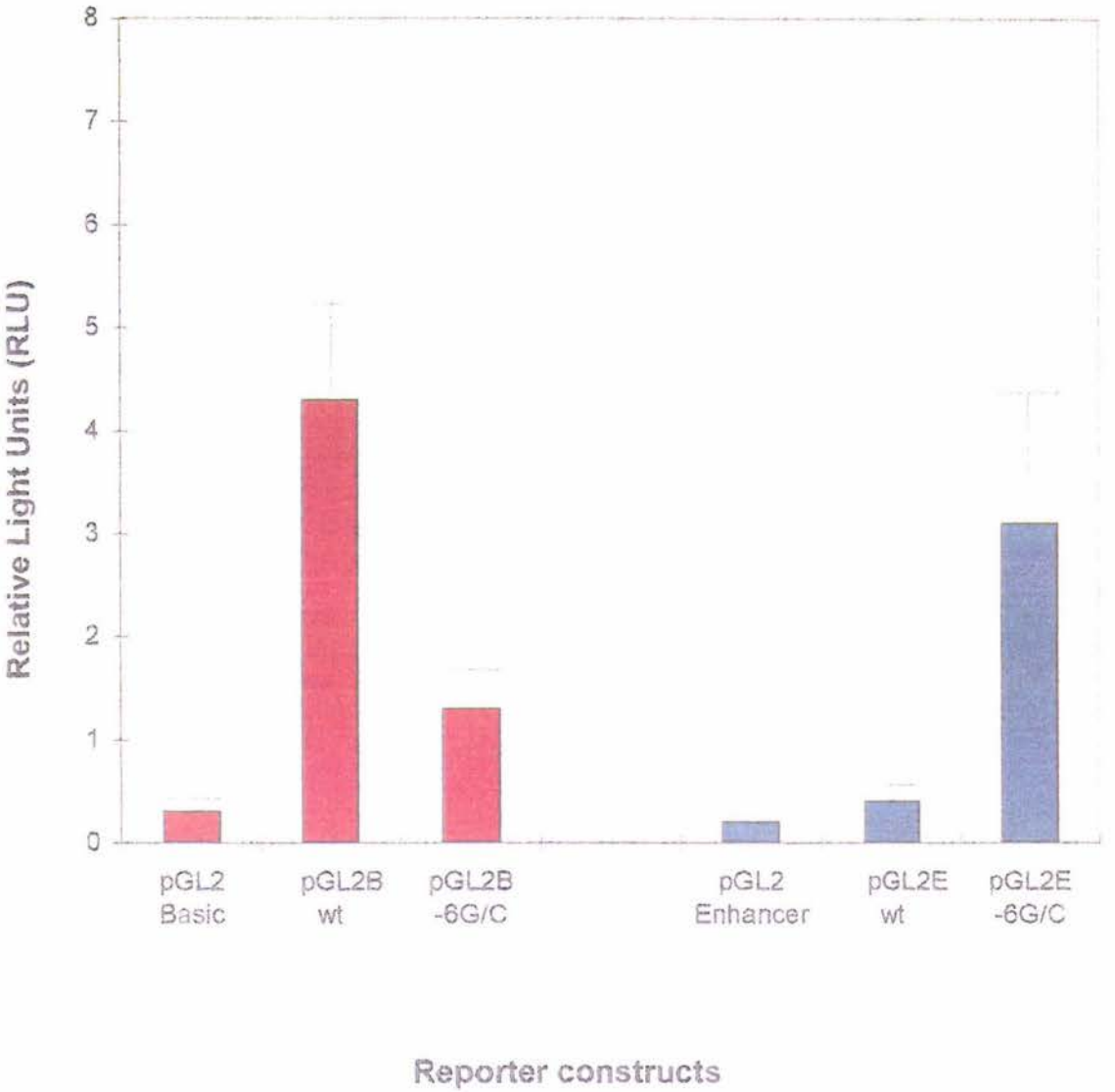


Figure 14. A comparison of luciferase expression between both normal and mutant promoter (region -220 to +45) constructs when present in either the promoterless pGL2 Basic or pGL2 Enhancer vector. The two promoterless vectors, pGL2 Basic and pGL2 Enhancer were used as controls to determine the background levels of luciferase. The luciferase activity (measured in relative light units) given for each construct was the average of three independent experiments, each containing three replicates of each plasmid.

3.6.3 Discussion

The reporter gene assay results in Fig. 14 show that the background level of luciferase activity from the promoterless vector, pGL2 Basic were similar to those produced from the pGL2 Enhancer vector.

A 3.3 fold reduction in promoter activity was observed between the normal and mutant (-6 G to C) promoter (-220 to +45) constructs in the pGL2 Basic vector. In contrast to the pGL2 Basic vector, the promoter activity of the wild-type pGL2 Enhancer vector was inhibited to background levels when expressed in the Alexander cell line. At the same time, the mutant promoter in pGL2 Enhancer was elevated to levels similar to that observed with the normal promoter in pGL2 Basic. This made any comparison between the wild-type and mutant promoter constructs difficult and the extent to which the promoter mutation was downregulated could not be accurately assessed.

The inability of the wild-type promoter to be expressed within the Alexander cell line suggested that a regulatory element present within the enhancer vector may be interfering with the transcriptional machinery. These results indicated that the pGL2 Basic vector may be more reliable in assessing the activity of the factor IX promoter within the Alexander cell line. Further experimentation using the pGL2 Enhancer vector was discontinued due to the inability of the wild-type promoter to function significantly. Therefore all subsequent experiments were carried out in the pGL2 Basic vector.

3.7 THE ROLE OF HNF4 IN THE REGULATION OF FACTOR IX GENE EXPRESSION

3.7.1 Introduction

The liver-enriched transcription factor, HNF4, is a member of the steroid receptor superfamily (Nishiyori *et al.*, 1994; Sladek *et al.*, 1990 cited by Kritis *et al.*, 1996) and is one of several proteins likely to be involved in the tissue-specific transcription of the factor IX gene. The binding of HNF4 to the factor IX promoter is required for constitutive promoter expression (Crossley and Brownlee, 1990; Crossley *et al.*, 1992; Kurachi *et al.*, 1994), enabling stable levels of factor IX to be translated and haemostasis within the individual to be maintained.

HNF4 has been found to act as an enhancer of promoter activity and as a positive regulator of a large number of liver-specific genes (Costa *et al.*, 1989 cited by Nishiyori *et al.*, 1994; Kritis *et al.*, 1996) and is thought to play an important role in the regulation of factor IX gene expression. The binding of HNF4 to the factor IX promoter at region -15 to -30 could therefore be expected to enhance the activity of the promoter.

Naka and Brownlee (1996) have recently postulated that an HNF4 binding site exists within the +5 to -15 region of the factor IX promoter. Their *in vitro* studies showed that the disruption of the -6 region of the factor IX promoter by a -6 G to C mutation resulted in the inability of the HNF4 transcription factor to transactivate the promoter to a similar order of magnitude as the wild-type promoter, in the presence of HNF4. The presence of the putative HNF4 site located at region +20 to +45 of the factor IX promoter was not however analysed by Naka and Brownlee (1996) as this site was excluded in their reporter gene constructs. It is therefore possible that this putative HNF4 site may be involved in the regulation of the factor IX promoter by enabling HNF4 to interact at this site if the main HNF4 binding site is disrupted.

In order to examine the extent of transcriptional activation created by the binding of HNF4 to the factor IX promoter, it was essential that an optimised transfection system using HNF4 was established within the Alexander cell line. In general, cultured

cells utilised for *in vitro* studies may be deficient or low in transcription factors that are required for optimal promoter expression (Coyle *et al.*, 1994; Picketts *et al.*, 1994). Such deficiencies in a cell line could subsequently result from either the type of cell line used, the over-passaging of cells or the inappropriate use of nutrients required for cell growth. Consequently, the expression from the factor IX promoter may be affected to such an extent that the luciferase assays would not reflect the true *in vitro* situation. Several *in vitro* studies have already reported deficient levels of the CCAAT enhancer binding protein (C/EBP) within the human hepatoma cell line, HepG2 (Babiss *et al.*, 1987). It is therefore possible that the Alexander cell line may also be deficient in certain transcription factors that are required for optimal luciferase expression. This would not be unfounded as the expression from the luciferase reporter constructs in earlier experiments (Chapter 3.3.5.3) were lower than reports by Crossley and Brownlee (1990) who used the HepG2 cell line.

An experimental system was therefore established to determine whether the levels of the transcription factors present within the Alexander cell line were sufficient to produce optimal expression from the luciferase reporter constructs. An expression vector for the transcription factor HNF4 was used for this analysis.

3.7.2 Results

Alexander cells were transiently co-transfected with the normal and mutant promoter (region -220 to +45) constructs in the presence of varying amounts of the expression plasmid for HNF4, pMT2-HNF4 (Ladias *et al.*, 1993). Cell lysates were prepared and the luciferase activity measured. All transfections were carried out using the 15 mm sized tissue culture plates. The results are summarised in Fig. 15 and the data presented in Table 5.

Table 5. Luciferase activity in the Alexander cell line comparing both normal and mutant (-6 G to C) promoter constructs in the presence of varying amounts of HNF4. Each construct contained the region -220 to +45 of the factor IX promoter fused to the luciferase gene in the promoterless vector, pGL2 Basic. The standard deviation of the mean from 5 independent experiments is shown.

Reporter constructs	Amount of HNF4 expression plasmid [μg]	Relative luciferase activity ± S.D
pGL2 Basic	0	0
	0.5	0
	2.5	1.3 ± 0.5
	4.5	1.6 ± 0.2
	10	2.0 ± 0.3
pGL2 B wt	0	3.9 ± 1.4
	0.5	19.9 ± 4.6
	2.5	62.8 ± 13.4
	4.5	82.8 ± 11.5
	10	58.6 ± 9.3
pGL2 B -6 G/C	0	1.5 ± 0.5
	0.5	16.3 ± 3.6
	2.5	35.8 ± 10.4
	4.5	32.8 ± 4.7
	10	18.3 ± 4.2

All reporter gene assays are expressed as 'relative luciferase activity' whereby the luciferase activity of the control vector, pGL2 Basic, was converted to 0 %. The remaining results were adjusted accordingly.

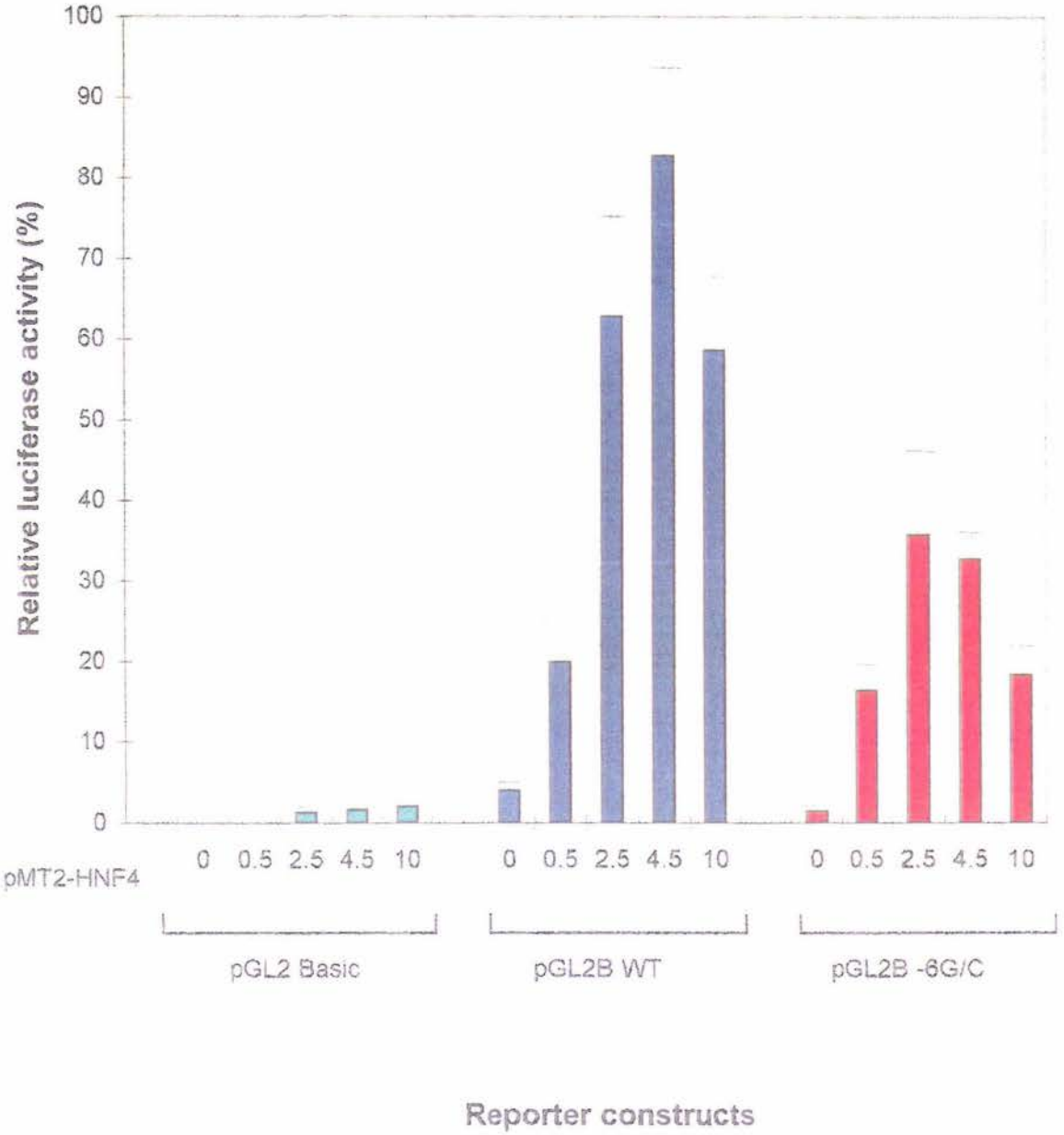


Figure 15. A comparison of the extent to which the normal and mutant (-6 G to C) promoter (region -220 to +45) constructs are transactivated in the presence of varying amounts of pMT2-HNF4. The promoterless vector, pGL2 Basic, was used as a control and the β-galactosidase encoding plasmid, pCH110 was used as an internal transfection control. The amount of luciferase is shown as a percentage relative to the plasmid pGL2 Basic , which was arbitrarily set at 0 %. All results are compiled from at least 6 independent experiments, each containing three replicates of each construct.

3.7.3 Discussion

Functional luciferase assays were carried out to discover the optimal range of transactivation of the normal and mutant promoter (region -220 to +45) constructs in the presence of HNF4.

The reporter gene assay results in Fig. 15, showed that the levels of luciferase activity produced from the wild-type and mutant promoter constructs were enhanced with increasing amounts of the HNF4 expression plasmid. Both wild-type and mutant promoter constructs produced similar levels of luciferase activity, within experimental error, when co-expressed with 2.5 μg and 4.5 μg of HNF4 plasmid. An initial 5 fold transactivation was observed with the wild-type promoter in the presence of 0.5 μg of pMT2-HNF4. The extent of transactivation from the wild-type promoter was subsequently increased with the addition of 2.5 μg and 4.5 μg of HNF4 vector and decreased slightly with the addition of 10 μg of pMT2-HNF4. The optimal level of transcription produced from the normal promoter construct was achieved with the presence of 4.5 μg of pMT2-HNF4 *i.e* a 21 fold increase in promoter activity was observed. This transactivation was similar to reports presented by Reijnen *et al.* (1992), whereby normal promoter (region -192 to + 38) constructs were transactivated ~20 fold in the HepG2 cell line in the presence of 2.5 μg of HNF4 vector. The elevation in luciferase activity with increasing amounts of the HNF4 expression vector suggests that HNF4 can bind and transactivate the factor IX promoter, supporting reports by Reijnen *et al.* (1992).

The mutant promoter construct followed a similar pattern of transactivation to that of the normal promoter construct, in the presence of exogenous HNF4. However, a higher level of transactivation was observed with the mutant -6 G to C promoter (region -220 to +45) construct in the presence of 2.5 μg pMT2-HNF4, whereby a ~24 fold increase in transactivation was observed. A ~1-2 fold difference in transcriptional activity still remained between the normal and mutant promoter constructs in the presence of 2.5 μg of pMT2-HNF4. This most likely reflected the inhibitory effects of the mutation on promoter activity due to the disabling of the unidentified transcription factor binding site at the nucleotide region +5 to -15.

The order of magnitude to which the -6 G to C mutation was downregulated in comparison to the normal promoter was found to decrease initially with increasing amounts of HNF4. For example, an initial 2.5 fold difference in promoter activity was observed between the normal and mutant promoters in the absence of exogenous HNF4. In the presence of 0.5 μg pMT2-HNF4 however, the difference between the normal and mutant promoters decreased to 1.2 fold. The extent of transcriptional downregulation between the wild-type and mutant promoter (region –220 to +45) constructs could therefore be affected by the concentration of transcription factor present within the cell line. Because of the similarity in transcriptional expression resulting from the addition of 2.5 μg and 4.5 μg of HNF4 vector with normal and mutant promoter constructs, future reporter gene assays were carried out with the addition of 2.5 μg of the HNF4 expression vector. The addition of 2.5 μg of pMT2-HNF4 instead of 4.5 μg of pMT2-HNF4 would also reduce the amount of HNF4 utilised in each experiment and effectively reduce the time and costs involved in preparing the expression plasmid.

The optimised experimental system established within the Alexander cell line was also used to assess the effects of the -6 G to C mutation on the binding of the HNF4 transcription factor to the factor IX promoter. Several reports have presented conflicting evidence concerning the interaction of the HNF4 transcription factor and the -6 region of the promoter. Mutations at the -6 region of the promoter have been suggested by Naka and Brownlee (1996) to interfere with the binding of the HNF4 transcription factor to the factor IX promoter. Expression of the normal and mutant (-6 G to C) promoter (region -200 to +40) CAT-constructs in HepG2 cells in their studies resulted in a ~2.1 fold difference between the activities of the normal and mutant promoters in the presence of 2 μg of the expression vector, pMT2-HNF4. This difference was similar to the studies shown in Fig. 15 in which both the -6 G to C and normal promoter constructs were transactivated in the presence of pMT2-HNF4. In contrast to reports by Naka and Brownlee (1996), the results in Fig. 15 illustrate the ability of the HNF4 transcription factor to bind and transactivate the factor IX promoter carrying a -6 G to C mutation to a level relatively higher than the optimal transcription obtained with the normal promoter. This suggested that the -6 G to C region did not disrupt the neighbouring HNF4 binding site at region -15 to –30 as suggested by Naka and Brownlee, 1996. It is possible that

Naka and Brownlee (1996) may have over-interpreted their results, as their data was similar within experimental error to the current studies presented here. For example, the normal promoter in their report was transactivated ~27 fold in the presence of HNF4 in comparison to the -6 G to C mutation which was transactivated ~18 to 22 fold. The data presented by Naka and Brownlee (1996) was not quantitative and the results represented by histogram were ambiguous and inconclusive. Taking into consideration the experimental error that was presented in their data, the *in vitro* experiments carried out by Naka and Brownlee (1996) did not clearly indicate that the differences between the normal and mutant promoters were a direct result of the disruption of the HNF4 site by a mutation at the -6 position of the factor IX promoter. If the -6 G to C mutation abolished the adjacent HNF4 binding site, then transactivation of the mutant promoter would be expected to be significantly lower than that observed for the wild-type in the presence of HNF4.

In the present study, the ability of the HNF4 transcription factor to transactivate the mutant promoter to a similar order of magnitude to that of the normal promoter, suggests that the -6 G to C mutation does not interfere with the binding of HNF4 to the promoter. It is possible that the observed increase in the mutant promoter expression may be due to the ability of the HNF4 transcription factor to bind elsewhere on the factor IX promoter. The incorporation of the putative HNF4 site at +20 to +45 as well as the previously identified site at -15 to -30 may have therefore resulted in the observed increase of the mutant promoter construct.

3.8 ANALYSIS OF PUTATIVE HNF4 BINDING SITES.

3.8.1 INTRODUCTION

Analysis of the factor IX promoter has identified a putative HNF4 binding site at region +20 to +45 of the promoter due to the homology between this region and the consensus DNA binding sequence for HNF4 (Pang *et al.*, 1990). Previous *in vitro* experiments have either excluded or potentially disabled this putative binding site in reporter gene constructs, which as a consequence may have affected the ability of HNF4 to bind and enhance promoter activity. The ability of the HNF4 transcription factor to increase expression from mutant promoters that disrupt the main HNF4 binding site at region -15 to -30, suggests that HNF4 may be binding elsewhere on the factor IX promoter.

The putative HNF4 binding site that lies at region +20 to +45 has not yet been analysed by *in vitro* studies. Therefore, a double mutation within the factor IX promoter (region –220 to +45) was created so as to disrupt the putative HNF4 site at region +20 to +45. The effect of this mutation was compared with the vector pGL2B -6 G to C, as well as the control vectors, pGL2B -26 G to C and pGL2 B -20 T to A, within the Alexander cell line.

3.8.2 Results

Alexander cells were transfected with the normal and mutant promoter (region -220 to +45) constructs in the presence and absence of the HNF4 expression vector, pMT2-HNF4. Transient transfections were carried out in the 15 mm tissue culture plates. Cell lysates were prepared and the luciferase activity for each sample was measured. A summary of the results is presented in Fig. 16 and the data presented in Table 6.

Table 6. Luciferase assay in Alexander cells comparing normal and Leyden promoters. Each construct contained the region -220 to +45 of the factor IX promoter fused to the luciferase gene in the promoterless vector, pGL2 Basic. The standard deviation of the mean from 6 independent experiments is shown.

Reporter constructs	Relative luciferase activity (%)	
	HNF4 expression plasmid	
	-	+
Normal	100 ± 16.5	321.2 ± 23.3
Leyden (-26 G to C)	13.2 ± 4.1	62.6 ± 16.8
Leyden (-20 T to A)	42.2 ± 9.9	159.0 ± 33.1
Leyden (-6 G to C)	33.5 ± 6.7	138.7 ± 28.7
Leyden (+26 G to C)	109.4 ± 12.9	403.7 ± 30.5
pGL2 Basic	6.0 ± 1.0	10 ± 2.2

All reporter gene assays are expressed as a 'relative luciferase activity' whereby the amount of luciferase is shown as a percentage relative to the wild-type promoter, which was arbitrarily set at 100. Results were averaged from 6 separate experiments, each of which contained triplicate transfections of all reporter constructs.

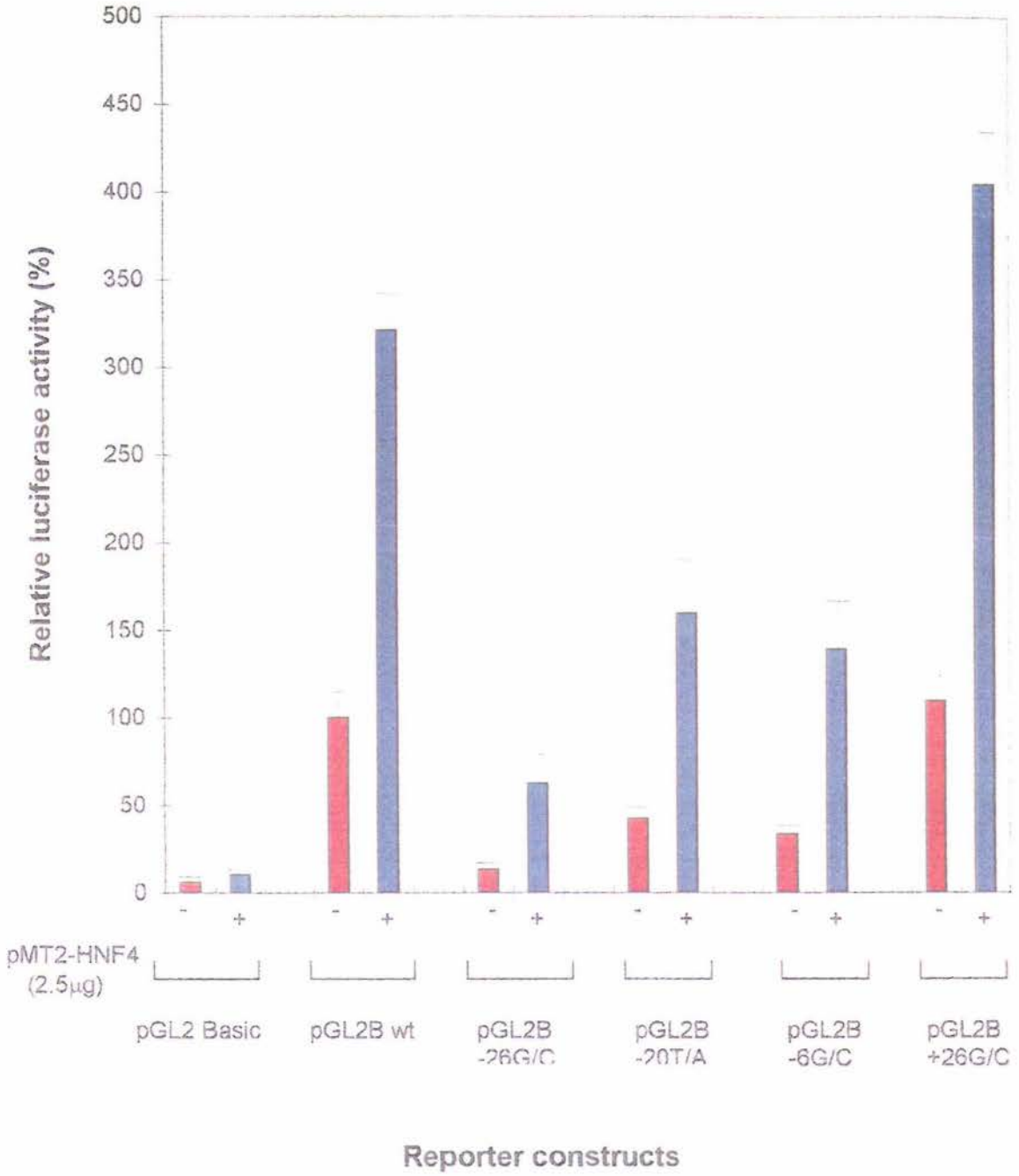


Figure 16. Analysis of the +20 to +45 region of the factor IX promoter. Both normal and mutant factor IX promoter (region -220 to +45) constructs were co-transfected in the presence and absence of the expression vector, pMT2-HNF4 (2.5 µg). The promoterless vector, pGL2 Basic was used an a negative control and the β-galactosidase encoding plasmid, pCH110 was used as a transfection control. The 'relative luciferase activity' was obtained by arbitrarily setting the luciferase assay results of pGL2B wt at 100 % and adjusting the other reporter assay results accordingly. The values given are the average of 6 independent experiments, each containing three replicates of each condition.

3.8.3 Discussion

Alexander cells were transfected with both normal and mutant promoter (region -220 to +45) constructs in the presence and absence of HNF4 to investigate the possibility of the HNF4 transcription factor interacting with a putative HNF4 binding site located at region +20 to +45.

Reporter gene assay results shown in Fig. 16 indicated that a mutation present within the +20 to +45 region of the factor IX promoter did not interfere with transcriptional activation from the promoter. In the absence of the HNF4 expression plasmid, the +26 G to C promoter (region -220 to +45) construct was transactivated to a similar order of magnitude as the wild-type promoter (region -220 to +45) construct. This was in contrast to the -26 G to C and -6 G to C promoter mutations, which resulted in a 7.6 fold and 3.0 fold (respectively) reduction in promoter activity. The -20 T to A mutant promoter (region -220 to +45) construct also exhibited a 2.4 fold reduction in luciferase activity when compared to the wild-type promoter in the absence of exogenous HNF4. A similar reduction in promoter activity was observed by Hirosawa *et al.* (1990) using the CAT-expression system. In their studies, the -20 T to A mutant promoter (region -416 to +4) construct was downregulated 3.6 fold to a relative CAT activity of 28.1 %. The slight discrepancy between the two studies may have been due to the extended promoter region utilised by Hirosawa *et al.* (1990) which may have altered transcription factor interactions.

In contrast to these reports, Reijnen *et al.* (1992), showed a marked decrease in promoter activity when the -20 T to A promoter (region -192 to +38) construct was expressed in HepG2 cells; *i.e.* a 17 fold decrease in activity was observed. In their later studies, Reijnen *et al.* (1993) showed that both the -20 T to A and -26 G to C promoter (region -192 to +38) constructs were further reduced to background level, similar to that of their promoterless control vector. The figurative representation of results illustrated by Reijnen *et al.* (1992, 1993) in their studies, was however ambiguous, and the background levels exhibited by both promoterless and mutant vectors were hard to decipher. The absence of error bars in their data also presented an overall picture of ambiguity.

Lower levels of promoter activity were also observed by Crossley *et al.* (1992) with the -20 T to A mutant promoter (region -189 to +21) when expressed in HepG2 cells. A 26 fold reduction in promoter activity was observed using the -20 T to A CAT-reporter construct in comparison to the wild-type promoter. Expression of the -26 G to C construct in their studies resulted in a further promoter reduction of 34 fold to a relative CAT activity of 2.9 %. This marked decrease in promoter activity was substantial when compared to the ~7.6 fold reduction in luciferase activity shown in Fig. 16.

The substantial differences in transcriptional activity resulting from the -20 T to A and -26 G to C mutations between the data presented here and other *in vitro* studies may be a direct consequence of the size of the factor IX promoter region utilised in each study. The different promoter regions incorporated into each reporter construct may alter protein interactions with the promoter region by excluding or incorporating various elements or recognition binding sites. Consequently, the transcriptional levels attained from the different promoter regions would be altered, and the effects of various mutations on the promoter may either be enhanced or masked. Alternatively, the different levels of transcription factors present within each cell line may have influenced the extent to which the factor IX promoter was regulated.

Co-expression of the reporter constructs in the presence of the expression vector pMT2-HNF4 resulted in the transactivation of all promoter constructs (Fig. 16). The wild-type promoter (region -220 to +45) construct was transactivated ~3 fold in the presence of HNF4. Surprisingly, both the -20 T to A and -26 G to C promoter (region -220 to +45) constructs were also transactivated in the presence of exogenous HNF4. The -20 T to A and -26 G to C mutations which disrupt the binding site for HNF4 at region -30 to -15 of the factor IX promoter both resulted in a ~4 fold increase in promoter activity in the presence of HNF4. This transactivation was similar to the transactivation of the wild-type promoter with HNF4 within experimental error. The latter result was unexpected as other *in vitro* studies had shown that the -26 G to C mutation was unable to promote transcription even in the presence of HNF4.

It is possible that the increases in promoter activity observed with the -20 T to A, -26 G to C and -6 G to C mutant promoter (region -220 to +45) constructs in the presence of exogenous HNF4 may be due to the binding of HNF4 to the putative HNF4 site at region +20 to +45. This could explain the reduction in transcription of mutations in other reports that exclude this site.

Reijnen *et al.* (1992) also reported an increase in promoter expression with the -20 T to A promoter (region -192 to +38) construct in the presence of HNF4. Although the mutant promoter was reported to be transactivated to a "limited" extent within HepG2 cells, the data presented by Reijnen *et al.* (1992) showed a ~83 fold increase in promoter activity in the presence of HNF4. The extent of this transactivation was considerably higher than the 27 fold increase observed with the wild-type promoter. Although a 4 fold difference remained between the mutant and normal promoter activities, the extensive increase in transactivation of the mutant promoter with HNF4 in their studies indicated that the HNF4 was transactivating the factor IX promoter.

In the presence of exogenous HNF4, both the wild-type and +26 G to C mutant promoter (region -220 to +45) constructs were transactivated 3.2 fold and 3.6 fold respectively. This result suggested that HNF4 may not be interacting with the +20 to +45 region of the factor IX promoter. Alternatively, it is possible that the mutation created within the factor IX promoter region was not strong enough to disrupt the HNF4 binding site and as a result the HNF4 transcription factor was able to bind and transactivate the promoter. The following model has been proposed by this author to explain the observed increases in transcription with the mutant -20 T to C, -26 G to C and -6 G to C promoter constructs in the presence of HNF4 (Fig. 17). This model is based on the assumption that a second HNF4 binding site exists within the factor IX promoter.

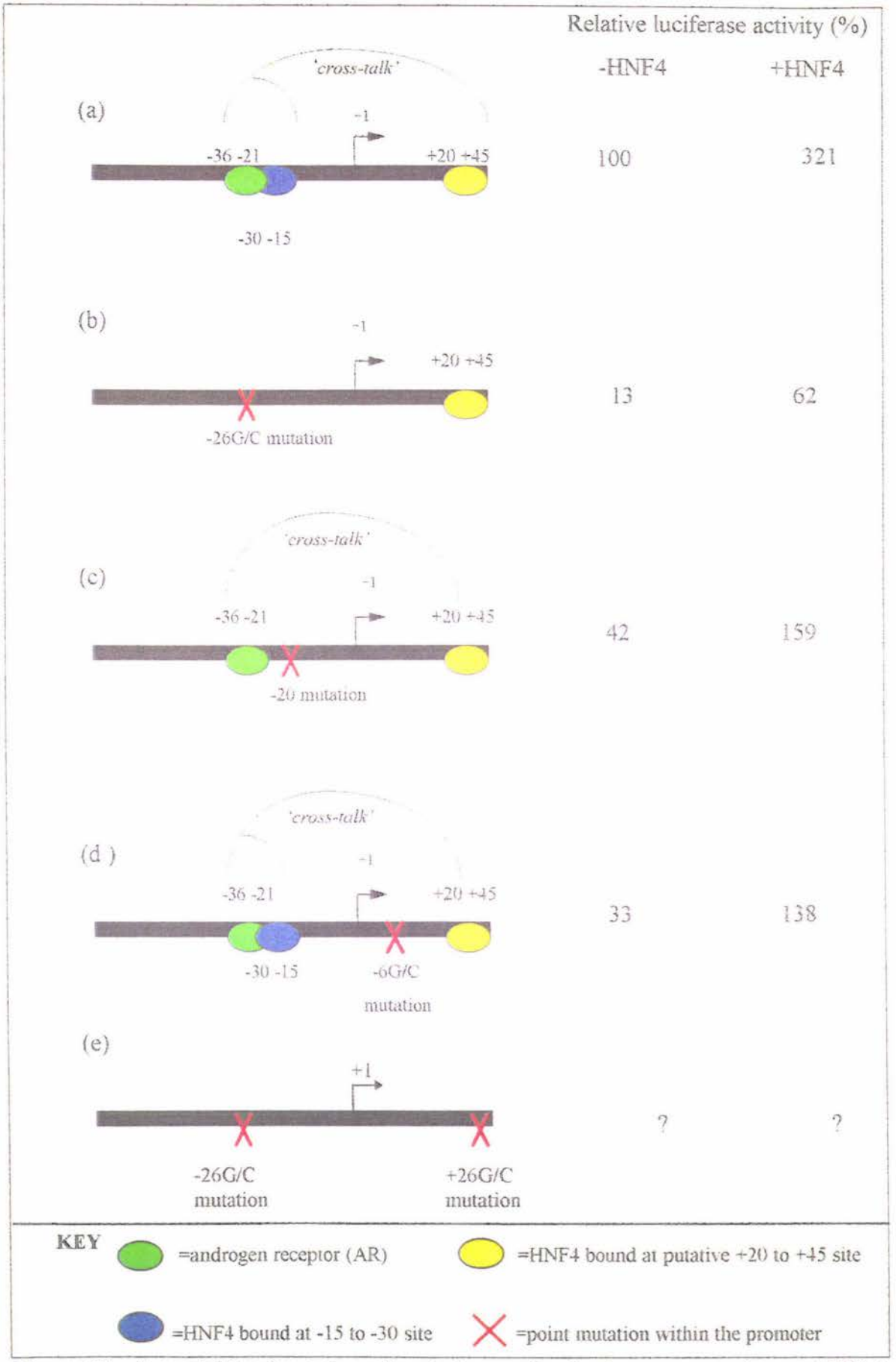


Figure 17. A model for the interaction between the Androgen receptor and HNF4 binding sites.

A: ***The normal factor IX promoter.***

Once bound to the promoter, the AR can interact directly or indirectly with HNF4 at either of the two HNF4 binding sites at -15 to -30 or +20 to +45 to promote efficient transcription.

B: ***The promoter with a mutation that disrupts the binding of the AR to its ARE, as well as disrupting the neighbouring HNF4 binding site at -15 to -30.***

The interaction between the AR and HNF4 transcription factors is disrupted. However, the HNF4 transcription factor is still able to bind to the putative HNF4 binding site at region +20 to +45. This interaction is not sufficient to restore normal promoter activity.

C: ***The promoter with a mutation that disrupts the binding of HNF4 to its binding site at position -15 to -30.***

The interaction between the AR and HNF4 transcription factors is maintained due to the binding of HNF4 to its putative site at +20 to +45. Because the putative site is weaker than the main HNF4 binding site at region -15 to -30, the levels of promoter activity are not optimal.

D: ***The promoter with a mutation that disrupts the binding of the unidentified transcription factor at region +5 to -15.***

The interactions between the AR and HNF4 transcription factors are maintained. However, these interactions are not sufficient to completely restore promoter activity if a mutation is present within the -6 region of the promoter.

E: ***Future research.***

The promoter with a mutation in both the ARE at position -36 and -21 and a mutation that disrupts the putative HNF4 binding site at +20 to +45.

This model suggests that the optimal expression produced from the factor IX promoter is achieved, in part, by a co-dependent interaction between the AR and HNF4 transcription factor bound to the two HNF4 binding sites, located at region -15 to -30 and region +20 to +45 (Fig. 17a).

A mutation present within the -26 region of the promoter would therefore disrupt all interactions between the AR and HNF4 transcription factor around the transcriptional start site, and as a consequence, result in the downregulation of the promoter (Fig. 17b). Subsequent enhancement of the promoter could only occur to a limited extent by the binding of HNF4 to its putative binding site at +20 to +45. This would account for the observed increase in the promoter activity reported with the -26 G to C reporter construct in the presence and absence of exogenous HNF4 (Fig. 17b).

The model also proposes that the putative HNF4 binding site at region +20 to +45 is weaker than the main HNF4 binding site at region -15 to -30. A mutation that disrupts the main HNF4 binding site (position -15 to -30) would result in the repressed binding of HNF4 to its second site at +20 to +45. This, in turn, would subsequently effect the interactions between the AR and HNF4 transcription factors as well as the overall outcome of promoter activity. This would explain the reported increase in transcription from the mutant (-20 T to A) promoter construct in the presence of exogenous HNF4, but the inability to restore the promoter activity to a normal level *i.e* as compared to the wild-type promoter (Fig. 17c). The inability of the AR and HNF4 transcription factor to compensate for a mutation within the -6 region of the promoter suggests that other factors are also required for optimal expression. However, it may be possible that the transient transfections carried out in this current study, required the addition of testosterone in order for the AR to bind to its respective site on the factor IX promoter. Further investigation into the possible interaction between the AR and HNF4 transcription factors would clarify the model proposed.

To support the hypothesis of 'cross-talk' between the AR and HNF4 transcription factors, a reporter gene construct could be prepared containing a double mutation that disrupts both the ARE and the putative HNF4 binding site (Fig. 17e).

This could then substantiate the suggestion that transcriptional activity is dependant upon the interaction of the AR and HNF4 transcription factors.

The results of the experimental system presented in Fig. 16 indicated that the HNF4 transcription factor could transactivate the factor IX promoter. The ability of HNF4 to transactivate the promoter containing mutations within the main HNF4 site at -15 to -30 suggested that the HNF4 may be binding elsewhere on the factor IX promoter. However, the inability of the +26 G to C mutation to downregulate transcription from the factor IX promoter indicated that HNF4 may not be interacting with the promoter at the +20 to +45 region. The existence of a putative HNF4 binding site located within the factor IX promoter (+20 to +45) cannot be ruled out simply on the basis of the *in vitro* studies shown here. It is possible that the HNF4 transcription factor may be binding elsewhere on the promoter if the main HNF4 binding site at region -15 to -30 is disrupted. Further assessment of the binding of HNF4 to the factor IX promoter region by footprinting, or mobility shift assays, would therefore be beneficial and would possibly clarify the *in vitro* experiments shown here.

3.9 THE ROLE OF THE CCAAT ENHANCER BINDING PROTEIN IN THE TRANSCRIPTION OF THE FACTOR IX PROMOTER

3.9.1 Introduction

The CCAAT enhancer binding protein (C/EBP) is one of several transcription factors that are expressed primarily within the liver. It is involved in numerous functions including cell proliferation, adipocyte differentiation and in the regulation of several immune-responsive genes (Chen *et al.*, 1997). C/EBP has also been implicated as one of the main transcription factors involved in the regulation of the factor IX gene (Chen *et al.*, 1997).

The C/EBP transcription factor belongs to a family of leucine zipper proteins that are characterised by highly homologous dimerisation and DNA binding domains (Jones, 1990; Scott *et al.*, 1992). Each of the family members contains a conserved

carboxyl terminal, otherwise known as a bZIP motif (Jones, 1990; Scott *et al.*, 1992) that is involved in the recognition and binding of DNA. Adjacent to the bZIP motif is a leucine zipper region that is required for the dimerisation of the protein (Jones, 1990; Scott *et al.*, 1992). The presence of the leucine zipper suggests that the members of the C/EBP family can interact with the DNA in either a homodimeric or heterodimeric manner.

Three isoforms of C/EBP were used in these studies. These were the C/EBPα, C/EBPβ and C/EBPδ. The three isoforms have been associated with the different regions of the factor IX promoter. For example, footprinting studies have shown that both the C/EBPα and C/EBPβ transcription factors bind well to the +1 to +18 and -199 to -219 regions of the promoter as well as binding weakly to the -77 to -99 region of the promoter (Picketts *et al.*, 1994).

In these current studies, the Alexander cell line was used to assess the extent of promoter transactivation in the presence of the three C/EBP isoforms. A titration experiment was carried out using the normal promoter (region -220 to +45) construct with increasing amounts of the expression vectors for the three C/EBP isoforms. To date, no similar work has been carried out within the Alexander cell line.

3.9.2 Results

Alexander cells were transiently transfected with the normal promoter (region -220 to +45) construct in the presence of varying amounts of the three C/EBP isoforms, C/EBPα, C/EBPβ, C/EBPδ. Cell lysates were prepared and assayed for luciferase gene activity. The results are presented in a histogram form (Fig. 18). All reporter gene assays are expressed as a 'relative luciferase activity' whereby the amount of luciferase is shown as a percentage relative to the normal promoter, which was arbitrarily set at 100. The results from the Alexander cell lines were averaged from five different experiments, each of which contained triplicate transfections of all reporter constructs.

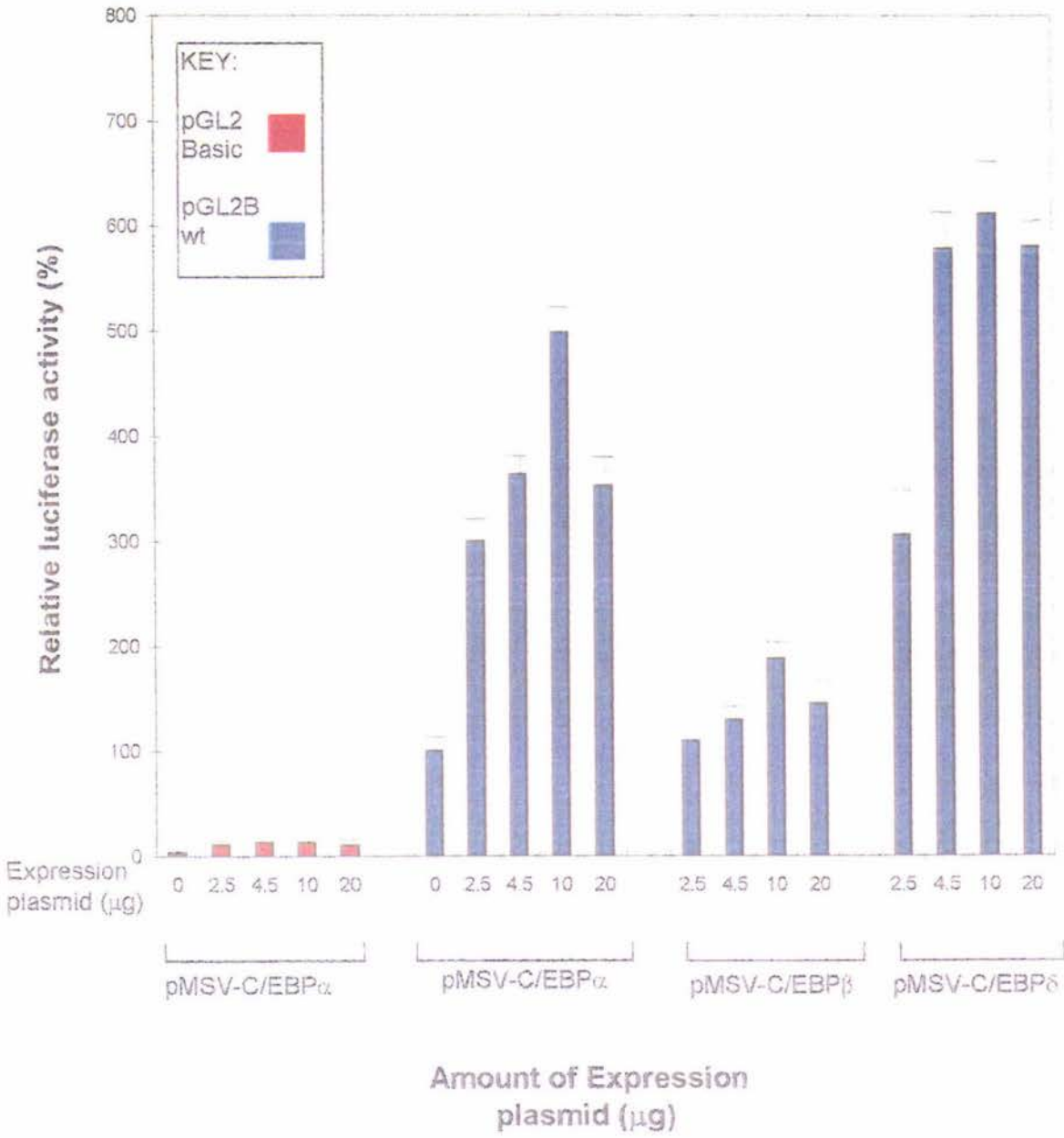


Figure 18 . A comparison of the extent to which the normal promoter (region -220 to +45) constructs is transactivated in the presence and absence of the three C/EBP isoforms; C/EBPα, C/EBPβ and C/EBPδ. The promoterless vector, pGL2Basic, was used as a control and the β-galactosidase encoding plasmid, pCH110 was used as a transfection control. The amount of luciferase is shown as a percentage relative to the plasmid pGL2B wt, which is arbitrarily set at 100. All results are compiled from at least five independent experiments, each containing three replicates of each construct. The standard deviation of the mean is shown. Extracts were prepared 48 hours after transfection and the luciferase and β-galactosidase activities measured.

3.9.3 Discussion

Functional luciferase assays were carried out to assess the extent to which the factor IX promoter could be transactivated in the presence of varying amounts of the three C/EBP isoforms: C/EBPα, C/EBPβ and C/EBPδ .

The reporter gene assay results (Fig. 18) showed a marked increase in transcription from the normal promoter (region -220 to +45) construct in the presence of both expression vectors, pMSV-C/EBPα and pMSV-C/EBPδ. Co-expression of the normal factor IX promoter (region -220 to +45) in the presence of 10 μg of pMSV-C/EBPα resulted in a 5 fold increase in promoter activity. This data was consistent with similar reports by Reijnen *et al.* (1994) using the CAT-reporter expression system. In their study, transient transfection of the normal promoter (region -189 to +21) construct into HepG2 cells with 15 μg of pMSV-C/EBPα resulted in a 4 fold induction in promoter activity. Further titration with the C/EBPα plasmid did not increase promoter activity. In contrast, experiments performed by Crossley and Brownlee (1990) using CAT-reporter constructs demonstrated a 30 fold increase in activity from the normal promoter (region -189 to +21) construct with 15 μg of C/EBPα. The discrepancies between the reports may be due to the differences in C/EBPα levels already present within the cell lines.

The promoterless control plasmid, pGL2 Basic, also exhibited a slight increase in luciferase activity when co-transfected with varying amounts of C/EBPα. This indicates that a cryptic C/EBP binding site may be present within the reporter plasmid. The slight increase in promoter activities with the control vector, pCATOO.1, had also been reported in similar experiments carried out by Reijnen *et al.* (1994).

The co-expression of the normal promoter (region -220 to +45) construct with increasing amounts of the expression plasmid, pMSV-C/EBPβ, did not have any profound effect on the transcription from the promoter. Footprinting studies performed by Picketts *et al.* (1994), using rat nuclear extracts showed that the C/EBPβ protein could interact with the factor IX promoter at regions +1 to +18 and -199 to -219. It is

therefore possible to speculate that the C/EBPβ transcription factor can bind to the factor IX promoter but may not have a transactivational role. Although the C/EBPβ transcription factor does not transactivate the human factor IX promoter, it is most likely that it binds to the same sites as C/EBPα and C/EBPδ as the DNA binding domains are similar in structure. It is also possible that C/EBPβ may have other physiological roles that have yet to be discovered.

The results in Fig. 18 show that the co-expression of the normal promoter with the C/EBPδ transcription factor transactivated the promoter to the greatest extent *i.e* the factor IX promoter was transactivated by ~6 fold in the presence of 10 μg of C/EBPδ. Footprinting studies performed by various groups including Picketts *et al.* (1994) have not shown the binding sites for the transcription factor C/EBPδ relative to the factor IX promoter. The data presented here clearly indicates that the C/EBPδ transcription factor is capable of binding and transactivating the factor IX promoter.

The optimal promoter activities in these studies was obtained with 10 μg of both expression plasmids for the C/EBPα and C/EBPδ transcription factors. The decrease in normal promoter activity with 20 μg of pMSV-C/EBPα and 20 μg of pMSV-C/EBPδ indicated a possible saturation of the promoter with excess transcription factor. The marked increase in promoter activity with the C/EBPα and C/EBPδ expression vectors indicated the importance of the two transcription factors in promoter activity. C/EBPα is usually present in low levels in human hepatoma cell lines, but is expressed in high levels within mitotically quiescent hepatocytes where it has been postulated to regulate various hepatic-specific genes (Scott *et al.*, 1992).

C/EBPα has also been suggested to be one of the main tissue-specific regulators of the factor IX gene (Picketts *et al.*, 1994). The binding of C/EBPα close to the transcriptional start site of the factor IX promoter suggests that it may be involved in the transcriptional initiation of the promoter (Crossley and Brownlee, 1990). Picketts *et al.* (1994) suggested that C/EBPα may be involved in establishing the basal transcription of the factor IX promoter early in development. In analogous experiments, Cao *et al.* (1991) analysed the specific roles of the different C/EBP isoforms in the development of

adipocytes. In their studies, the three C/EBP isoforms were expressed sequentially during adipocyte differentiation. Both the C/EBPβ and C/EBPδ isoforms were expressed early in development with the induction of adipogenic hormones, prior to the C/EBPα expression. The levels of the C/EBPβ and C/EBPδ isoforms decreased before the onset of C/EBPα expression. The regulatory cascade described by Cao *et al.* (1991), which involved three C/EBP isoforms during adipocyte development may also be present within the factor IX promoter. Interestingly, Cao *et al.* (1991) also demonstrated that the C/EBPβ and C/EBPδ isoforms were capable of forming heterodimers with one another as well as with C/EBPα.

Footprinting studies by Picketts *et al.* (1994) also indicated the possible homo- or heterodimeric interaction between the C/EBP family members prior to their binding to the factor IX promoter. It may be possible to speculate that the C/EBPβ and C/EBPδ isoforms may heterodimerise with each other prior to the induction of C/EBPα which may be involved in creating the basal transcription of the factor IX promoter by binding close to the start site. The ability of the C/EBP family members to dimerise with each other and perhaps with other leucine zippers provides a wide range of opportunities for regulation (Jones, 1990).

These studies clearly demonstrate the transactivation of the factor IX promoter by the transcription factors C/EBPα and C/EBPδ and suggest that they may play an important role in gene expression within the liver. The differences in the transcriptional levels created by the different members of the C/EBP family suggest that different recognition and binding mechanisms may be employed by each member. Although the C/EBP family have closely related DNA binding domains (Lamb and McKnight, 1991), the differences between the DNA-binding domains of each of the C/EBP members may specify the different affinities for the promoter and thus the extent to which the promoter is transactivated. The studies presented here will enable further assessment of other C/EBP sites that may exist within the factor IX promoter.

3.11 FACTOR IX - AN ACUTE PHASE PROTEIN?

3.11.1 Introduction

The acute-phase response of the inflammatory system refers to the physiological changes that occur within the body after stimulation during infection or physical trauma (Poli *et al.*, 1990; Steel and Whitehead, 1994). The acute phase response is initiated and co-ordinated by several mediators that are released from the cell (Fig. 19). Such mediators include phylatoxins, glucocorticoids and cytokines such as the tumour necrosis factor-α (TNF-α), interleukin-1β, and interleukin-6 (Steel and Whitehead, 1994; Citarella *et al.*, 1997). As a result of the release of mediators within the body, the biosynthesis of the liver is changed (Steel and Whitehead, 1994). Consequently, the steady-state concentration levels of plasma proteins produced from the liver are altered shortly after the onset of infection or tissue-damage (Steel and Whitehead, 1994; Citarella *et al.*, 1997). The concentration and the rate of synthesis to which the acute-phase plasma proteins are produced within the liver are dependant upon the type of mediator that induces protein synthesis (Poli *et al.*, 1990; Citarella *et al.*, 1997). Both positive and negative changes in the concentration of plasma protein are caused by the alteration of transcription from genes that encode acute-phase proteins (Poli *et al.*, 1990; Citarella *et al.*, 1997). Several cytokines have been shown to regulate gene transcription by activating various nuclear factors such as NF-IL6/CCAAT-enhancer binding protein β (C/EBPβ), NF-IL6/CCAAT-enhancer binding protein δ (C/EBPδ) and hepatocyte nuclear factors (HNFs), that interact with cis-acting elements present within the promoter region of the acute-phase genes (Steel and Whitehead, 1994). The cytokine interleukin-6 (IL-6) has been shown to regulate all of the acute-phase proteins (Poli *et al.*, 1990; Citarella *et al.*, 1997).

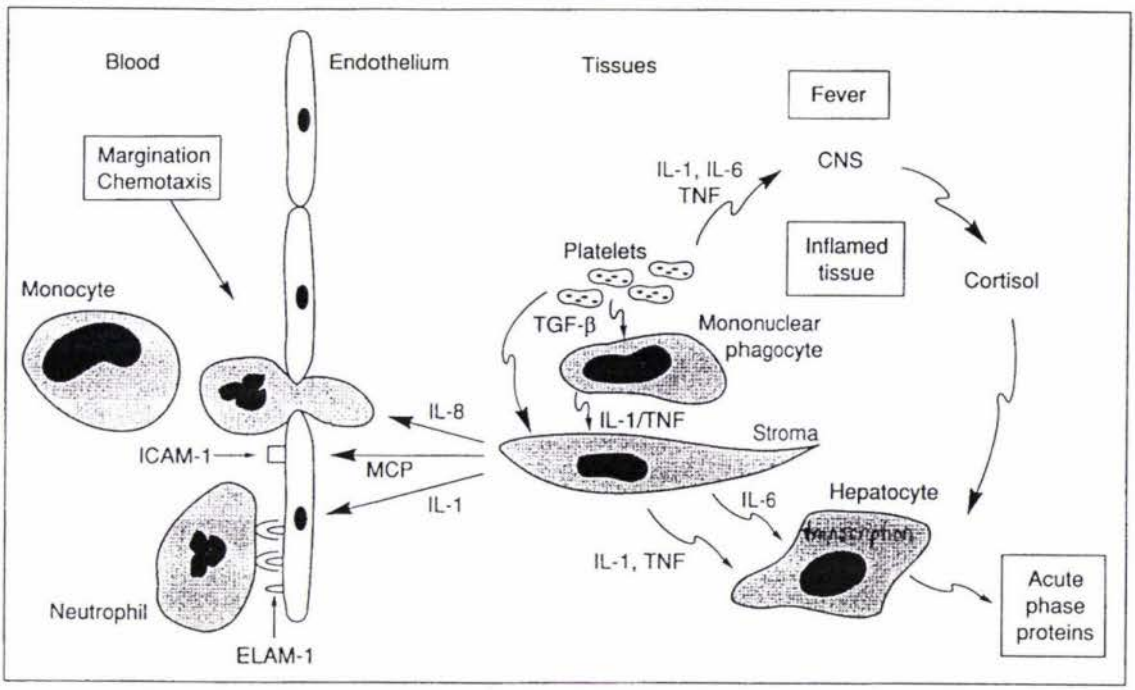


Figure 19. A schematic representation of the acute-phase response. The acute phase response is triggered by trauma and causes the release of mediators such as cytokines and glucocorticoids from the cell which initiate the acute-phase response. (Steel and Whitehead, 1994).

Interleukin-6 is a pleiotrophic cytokine that is involved in a wide range of biological activities related to the immune and inflammatory response (Akira *et al.*, 1990; Majello *et al.*, 1990). The 26 kDa glycoprotein is produced from various cell types which include fibroblasts, macrophages and T and B-lymphocytes (Majello *et al.*, 1990) during infection or trauma. Interleukin-6 has been shown to increase the expression of genes coding for the positive acute-phase proteins such as the haptoglobin, haemopexin and C-reactive protein genes and downregulate the negative acute-phase proteins (Akira *et al.*, 1990; Majello *et al.*, 1990; Citarella *et al.*, 1997).

In vitro cultured cell lines have been shown to respond to IL-6 by increasing or decreasing the rate of transcription of several acute–phase genes. The discovery of IL-6 responsive elements (IL-6 RE) within the promoters of several acute-phase genes had lead to identification of proteins that bind to the IL-6 REs of the acute-phase genes (Majello *et al.*, 1990). One of these proteins known as nuclear factor IL-6 (NF-IL6), has the ability to interact with several IL-6 inducible promoters. This transcription factor,

which is expressed constitutively in human hepatoma cell lines such as HepG2 and HepG3, has also been shown to be augmented in the cell line by the addition of IL-6 (Akira *et al.*, 1990). It is therefore possible that the effect of IL-6 on the transcription from promoters containing IL-6 REs may be mediated by the action of the NF-IL6 protein.

Interestingly, NF-IL6 shares a high sequence homology to the transcription factor C/EBP in its DNA binding domain (Osada *et al.*, 1996). The ability of the C/EBP transcription factor to interact with the factor IX promoter at region +5 to +15 suggests that NF-IL6 too, may be able to bind to the factor IX promoter. It could also be speculated that the factor IX protein could be involved in the acute-phase response to inflammation as a plasma protein induced by cytokine action. This would not be an unfounded suggestion as both factor VIII and factor XI proteins have been implicated in the acute-phase response. Recent studies carried out by Citarella *et al.*, (1997) have also shown that the synthesis of a factor XII protein is downregulated by the presence of interleukin-6 when expressed within HepG2 cells.

Therefore the response of the factor IX promoter to IL-6 was tested in the Alexander cell line. The ability of IL-6 to alter expression from the factor IX promoter would therefore implicate the factor IX protein as a possible acute-phase protein involved in the inflammatory response. To date, no similar work has been performed with the factor IX promoter.

3.11.2 Results

The Alexander cell line was transfected with the normal promoter (region -220 to +45) construct in the absence of interleukin-6. After 24 hours, the media was exchanged and the appropriate concentrations of the cytokine, interleukin-6 were added to the media. After a further 24 hour incubation period, cell lysates were prepared and the luciferase activity for each sample was measured (Table 7).

Table 7. Levels of luciferase and β-galactosidase activity obtained from the first transfection within the Alexander cell line. The standardised activity was calculated by dividing the luciferase activity by the β-galactosidase activity.

Reporter construct	Amount of IL-6 (ng)	Luciferase Activity	β-galactosidase Activity	Standardised Activity
pGL2 Basic	0	0.110	0.423	0.260
	1	0.120	0.333	0.360
	8	0.022	0.133	0.165
	50	0.067	0.271	0.247
	80	0.056	0.230	0.243
	100	0.033	0.231	0.143
	120	0.074	0.281	0.263
pGL2B wt	0	0.378	0.153	2.471
	1	0.513	0.200	2.565
	8	0.441	0.120	3.675
	50	2.703	0.312	8.663
	80	2.786	0.189	14.741
	100	3.472	0.396	8.768
	120	2.962	0.238	12.445

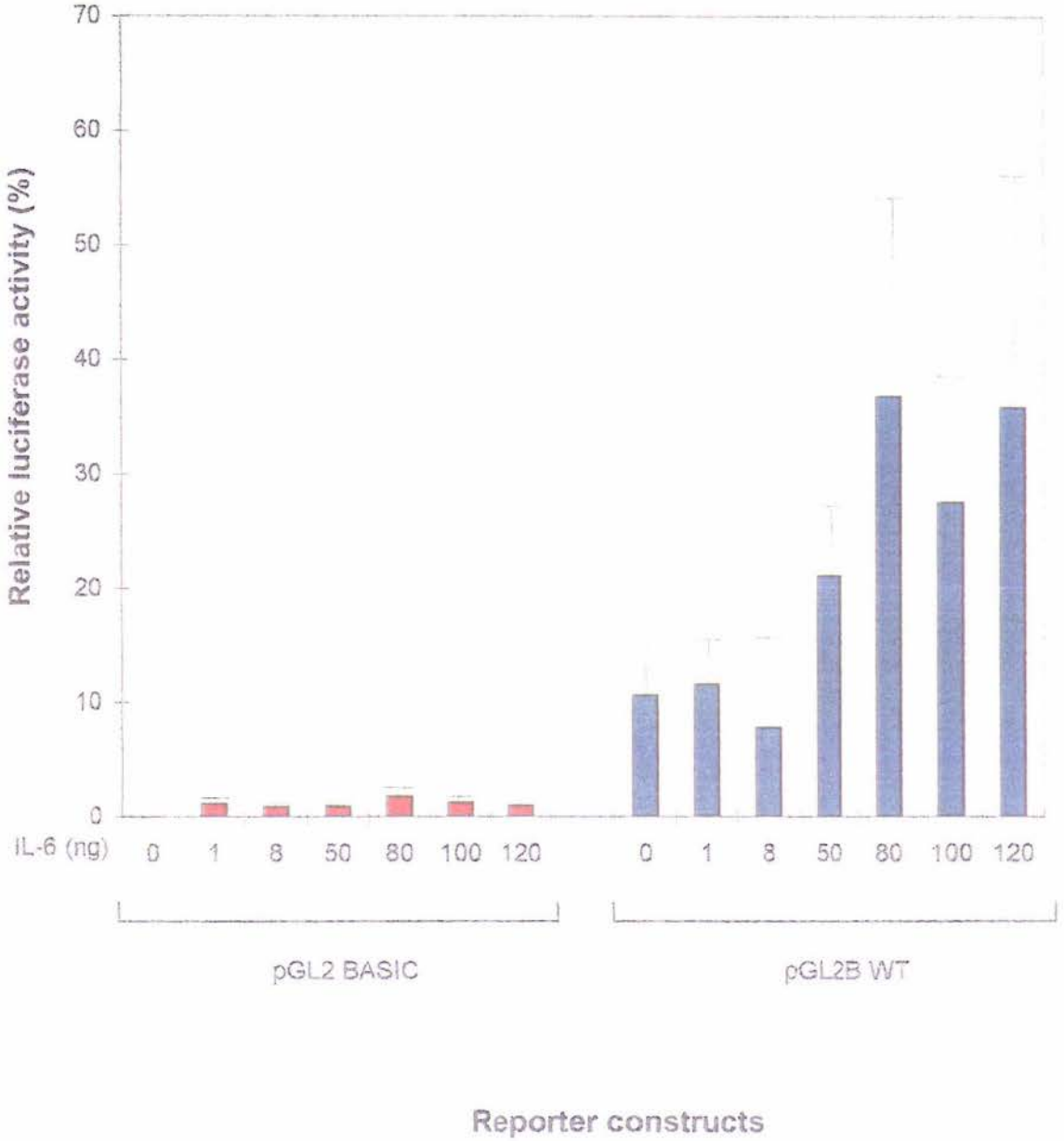


Figure 20. Transactivation of the factor IX promoter by the addition of IL-6. Alexander cells were transiently transfected with the promoterless pGL2 Basic vector and a wild-type promoter that contained region -220 to +45 of the factor IX promoter. Twenty-four hours after transfection, the cells were washed and treated with varying amounts of IL-6 (ng). The amount of luciferase is shown as a percentage relative to the plasmid, pGL2B wt, which is arbitrarily set at 100. All results are compiled from at least 7 separate experiments, each containing three replicates for each condition.

3.11.3 Discussion

In order to determine whether the factor IX protein may be involved in the acute phase response, varying concentrations of interleukin-6 were incorporated into luciferase gene assays with the wild-type promoter. Reporter gene assays presented in Fig. 20 showed that wild-type promoter construct could respond to IL-6 giving transcriptional activations between 3 to 6 fold higher than in the absence of IL-6. The results were extremely variable and difficult to reproduce. Figure 20 represented data collected in preliminary experiments, therefore no firm conclusion could be drawn about the response of the factor IX promoter to IL-6.

The fluctuation in promoter activity observed with increasing levels of IL-6 may have been the result of environmental conditions at the time when the experimentation system was established. For example, it is possible that the fetal calf serum within the media contained negative regulators that interfered with the binding of the transcription factor, NF-IL6 to the factor IX promoter. This would not be an unfounded hypothesis as Citarella *et al.* (1997) in analogous experiments, found that the fetal calf serum incorporated into their media interfered with the expression of factor XII production in HepG2 cells that had been stimulated with IL-6. The increased levels of the factor XII protein expressed in media stripped of fetal calf serum suggested that regulators within the serum may have interfered with IL-6 induction. Other nuclear factors that are either absent or deficient within the Alexander cell line may also be required for IL-6 induction of hepatic promoters (Majello *et al.*, 1990) and may explain, in part, the variance observed with the reporter gene construct assays presented here.

The transcriptional regulation of IL-6 responsive genes is a complex mechanism that may not be able to be represented with artificial promoters. The reporter gene assays presented in Fig. 20 have not been able to demonstrate clearly the induction of the factor IX promoter by the presence of interleukin-6. In order to determine whether the transcription factor NF-IL6 (which is stimulated by IL-6) can bind to the factor IX promoter, both mobility shift and footprinting assays would be required to clarify the luciferase assay results presented here.

3.12 ELECTROPHORETIC MOBILITY SHIFT ASSAYS

3.12.1 Introduction

The electrophoretic mobility shift assay (EMSA) is an *in vitro* method utilised for the analysis of protein interactions with a DNA sequence. In particular, the sequence specificity of a protein-DNA interaction can be assessed by analysing the extent to which the protein of interest binds to a promoter region. The extent of the interaction is reflected by the rate of mobility of the protein-DNA complex through a non-denaturing polyacrylamide gel. This technique is schematically represented in Fig. 21.

The radioactive labelling of a DNA fragment enables the interaction between protein and DNA to be monitored. This fragment can subsequently be incorporated into an incubation reaction with the cellular protein extract containing the protein of interest. A protein-DNA complex can then be separated from free DNA by means of a polyacryamide gel. The rate at which the protein-DNA complex migrates through the polyacrylamide gel will be slower than free DNA due to the comparative size of the complex, and will often appear as a shifted band of DNA at the top of the gel (Watson *et al.*, 1992).

3.12.2 Competition electrophoretic mobility shift assays

To determine the specificity of a protein-DNA interaction (*i.e* which bands on the EMSA are due to proteins binding specifically to a sequence), competition experiments are performed. These experiments involve the incorporation of unlabelled competitors, which may subsequently compete with the labelled DNA fragment for the binding of specific proteins found within the cell extracts. By increasing the concentration of the unlabelled competitor in the reaction, the specificity of the protein toward either the unlabelled or labelled DNA may also increase. The intensity of the resultant EMSA band would therefore reflect the amount of competition observed between the competitor DNA and the labelled DNA for the protein of interest. For example, if the protein of interest is more specific for the unlabelled competitor DNA

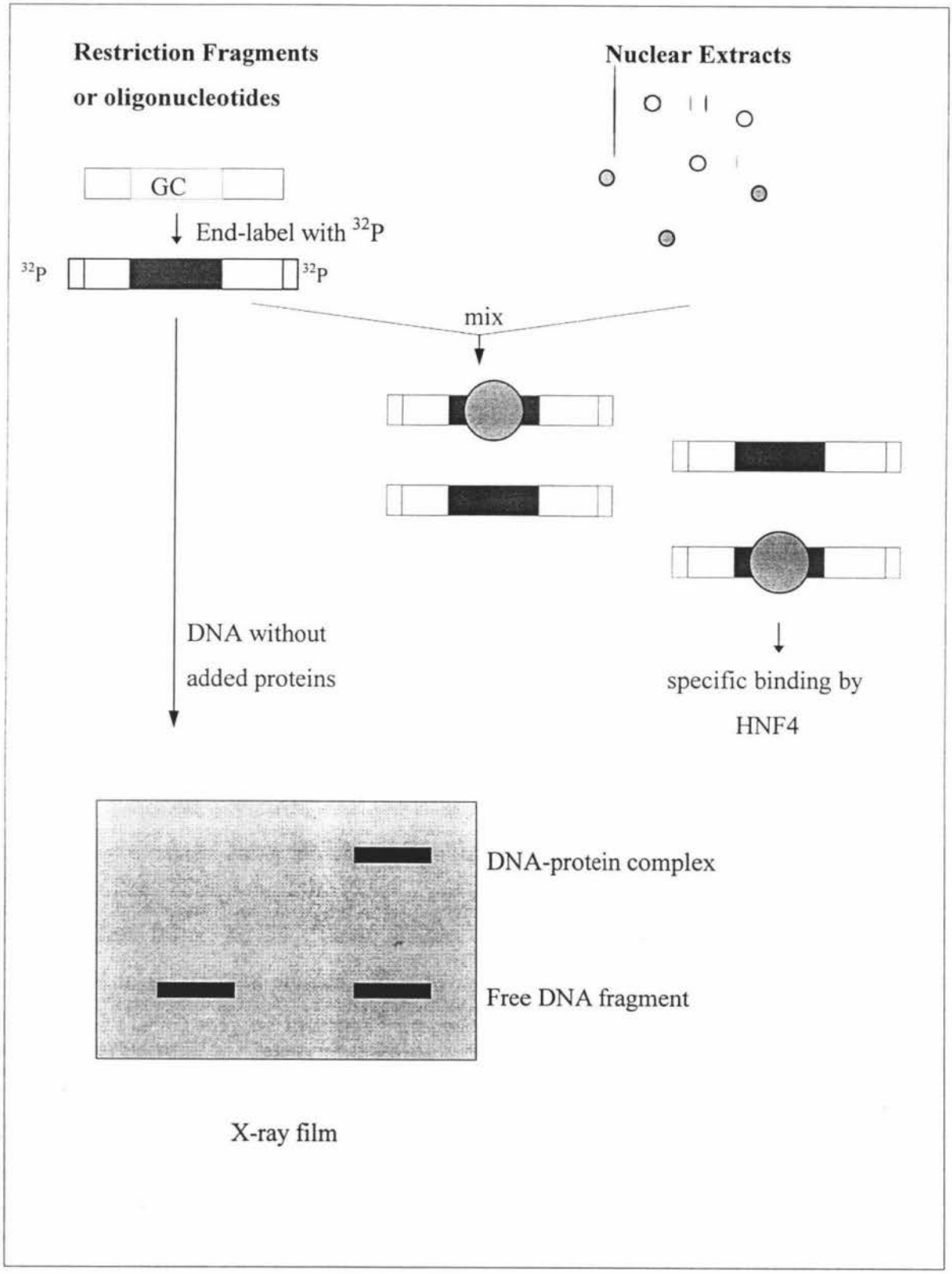


Figure 21. Schematic diagram of the electrophoretic mobility shift assay (EMSA). Modified from Watson *et al.* (1992).

than the labelled fragment, a decrease in the intensity of the band will be observed.

The competitor oligonucleotide, which is incubated with cellular extracts prior to the addition of the labelled DNA, may either be homologous (specific) or non-homologous to the labelled DNA. The addition of a homologous competitor as a positive control in the EMSA study would thus enable a comparison to be made between the extent of competition observed with the non-homologous competitors and the labelled DNA fragment.

The EMSA method provides direct evidence that a DNA element binds to a protein within a cell, therefore it may support other results from assays which may be inconclusive.

3.13 THE INTERACTION OF THE HNF4 TRANSCRIPTION FACTOR WITH THE FACTOR IX PROMOTER: AN EMSA STUDY

3.13.1 Introduction

Recent *in vitro* studies carried out by Naka and Brownlee (1996) have suggested that the +5 to -15 region of the factor IX promoter may in fact be a binding site for the transcription factor, HNF4. These studies conflicted with reporter gene assay results presented in the current studies and suggested that the report presented by Naka and Brownlee (1996) may be incorrect. In order to confirm the experiments presented in Chapter 3.3.7, the interaction of the HNF4 transcription factor with the factor IX promoter was studied by EMSA.

The cellular extracts utilised in the EMSA studies were prepared from a Cos 1 cell line (Simian monkey kidney fibroblast cells), and the radiolabelled oligonucleotide incorporated into each assay represented the α1-antitrypsin HNF4 site. Because the cellular extract contained numerous DNA-binding proteins that could interact non-specifically with the factor IX promoter, a non-specific competitor poly (dI-dC), was incorporated into each reaction to allow for the detection of specific binding. The

amount of poly (dI-dC) used to remove non-specific binding had already been determined. Preliminary EMSA studies were also carried out to determine the protein concentration of the cellular extract to ensure that sufficient protein was added to produce a detectable mobility shift assay (Fig. 22).

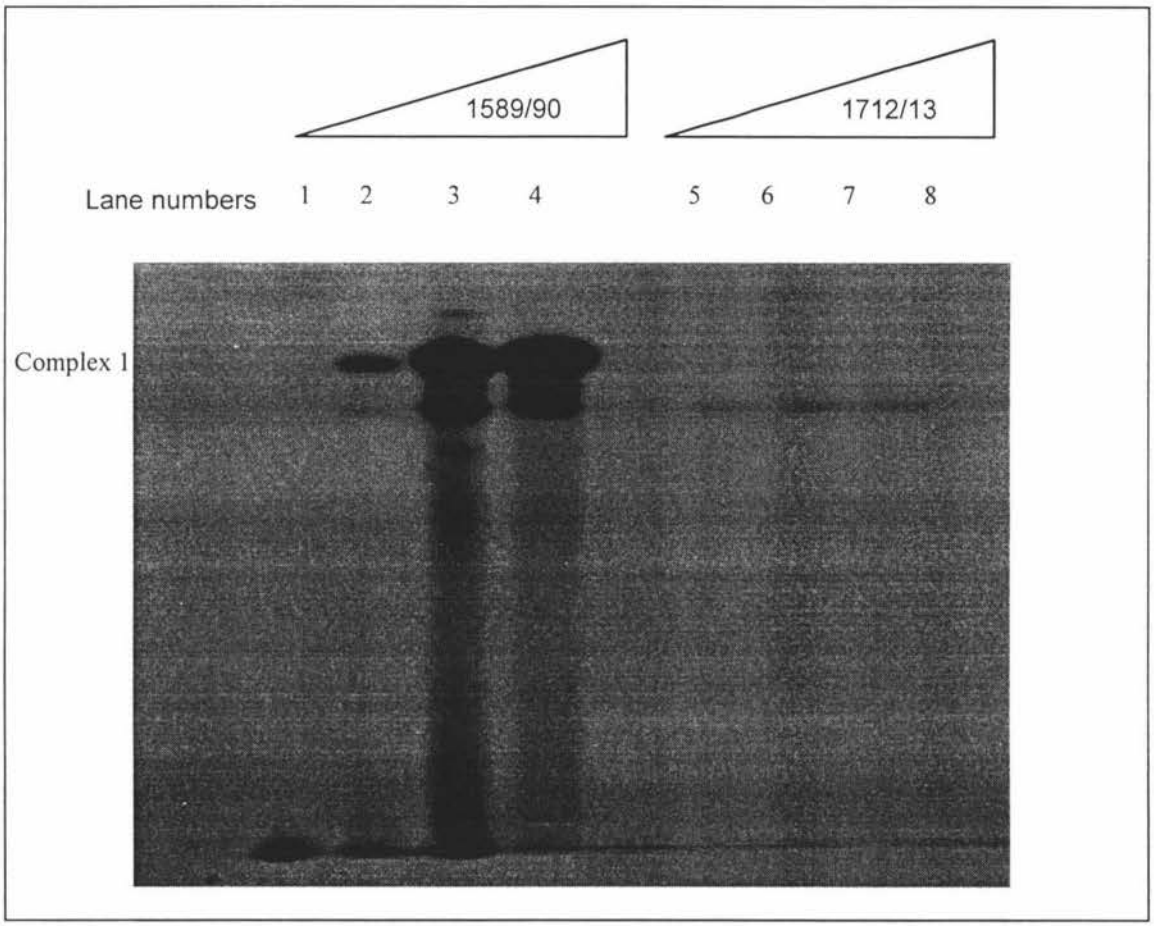


Figure 22. Determination of the protein concentration from the cellular extract. An incubation reaction was set-up containing 0.5 ng of ^{32}P labelled oligonucleotide representing either the HNF4 site for the α1-antitrypsin promoter (1589/90) or the oligonucleotide representing the factor IX promoter from region -14 to +6 (1712/13), 1 μg of poly (dI-dC) and varying amounts of Cos cell extracts. After incubation, the samples were subjected to electrophoresis on a 4 % polyacrylamide gel in 0.25 x TBE. Lanes 1 and 5 contained the control reaction, carried out in the absence of nuclear extract. Lanes 2 and 6 contained 1μl of Cos cell extract. Lanes 3 and 7 contained 2 μl of Cos cell extract. Lanes 4 and 8 contained 4 μl of Cos cell extract.

To establish which of the bands resulted from the binding of specific proteins to the factor IX promoter, competitor oligonucleotides were added to the EMSA reaction. All EMSAs were carried out several times to ensure reproducibility.

3.13.2 Results

Competition EMSAs using Cos 1-expressed HNF4 resulted in the formation of two protein-DNA complexes which are shown in Fig. 23. The two complexes produced a similar pattern of interaction.

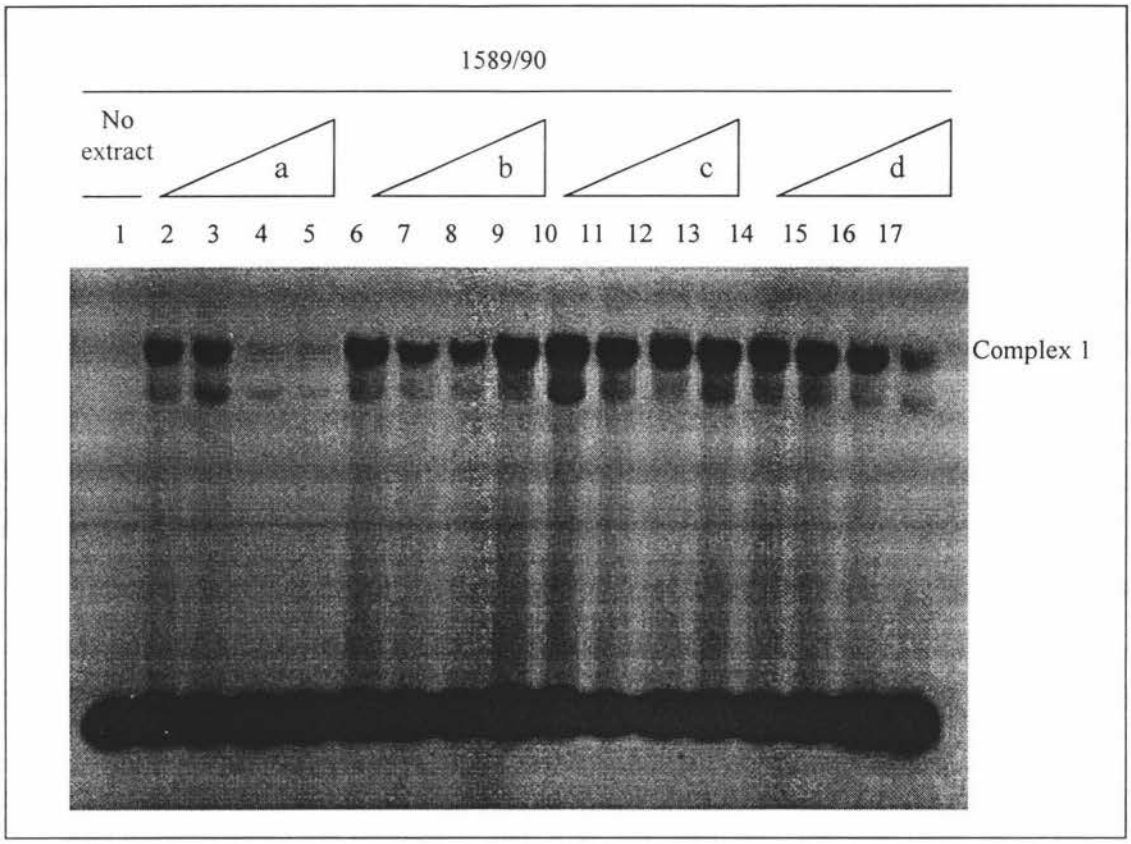


Figure 23. Competitor EMSA using Cos 1-expressed HNF4. An incubation reaction was set-up containing 0.5 ng of ^{32}P labelled oligonucleotide representing the HNF4 site for the α1-antitrypsin promoter, 1 μg of poly (dI-dC) and 1 μg of Cos 1 cell extract. After incubation, the samples were subjected to electrophoresis on a 4 % polyacrylamide gel in 0.25 x TBE. Lane 1 contained the control reaction, carried out in the absence of the nuclear extract. The amount of unlabelled DNA added as a competitor to the reaction mixtures was increased to 10, 100 and 200 fold excess over the labelled DNA. Competitor 'A' is the unlabelled homologous competitor, 1589/1590 representing the HNF4 site of the α1-AT promoter; competitor 'B' is the unlabelled oligonucleotide 1579/1580 representing region -40 to -9 of the factor IX mutation containing a -26 G to C mutation; competitor 'C' is the unlabelled oligonucleotide 1648/1649 representing region -23 to +6 of the factor IX promoter containing a -6 G to C mutation; and competitor 'D' is the unlabelled competitor 1712/1713 representing the factor IX promoter region from nucleotides -14 to +6. The assays in lane 2, 6, 10 and 14 did not contain competitor oligonucleotides. A second gel shift was carried out using 1712/1713 as a probe to see whether HNF4 was binding to this site. This gel shift showed that HNF4 was not capable of binding which supports the results shown here.

3.13.3 Discussion

To study the interaction of the HNF4 transcription factor with the factor IX promoter, EMSAs were carried out using Cos 1-expressed HNF4. Of the two complexes that were formed in the EMSA, only complex I appeared to be the result of specific binding of proteins to the labelled DNA. As expected, the protein (s) in complex I was able to bind to the homologous unlabelled HNF4 site of the α1-antitrypsin promoter when increasing concentrations of unlabelled competitor were incorporated into the assay. The competition observed between the unlabelled competitor DNA and the labelled HNF4 site of the α1-antitrypsin promoter for the HNF4 transcription factor was illustrated in the EMSA as a decrease in the intensity of retarded bands (Fig 23, lane 2 to lane 4) with increasing levels of unlabelled competitor.

The oligonucleotides representing the factor IX promoter containing either a -6 G to C or -26 G to C mutation were incorporated into the EMSA study as negative controls. Both mutations disrupt the HNF4 binding site between nucleotides -15 to -30 so therefore competition would not be expected. The unlabelled oligonucleotide representing the -14 to +6 region of the factor IX promoter was found to show very little specificity toward the HNF4 transcription factor when the levels of unlabelled competitor were increased in the assay (Fig. 23, lane 17).

The results presented in Fig. 23 were in general agreement with the functional assay in the Alexander cell line shown in Chapter 3.3.7.3. This illustrated that the -6 region of the factor IX promoter was not likely to be an HNF4 binding site.

CHAPTER FOUR: FINAL DISCUSSION

4.1 INTRODUCTION

The interaction of transcription factors with the factor IX promoter are critical for optimal gene expression and as a consequence could lead to a synergistic effect on transcription activation or repression (Jones, 1990). The various transcription patterns generated by the factor IX promoter could therefore be created by the various combinations of factors interacting with the promoter region (Jones, 1990). Such combinations could subsequently be altered by the size of the promoter region incorporated into each reporter gene construct for the *in vitro* analysis of promoter expression.

Discrepancies in the levels of factor IX promoter activity attained between the various research groups and the data presented in Chapter 3 may be attributed to the use of different sized promoter regions incorporated into the reporter gene constructs. By altering the size of the promoter either to incorporate or to eliminate various binding sites for transcription factors required for constitutive factor IX promoter expression, the overall protein-DNA interactions may be changed as a consequence, therefore producing an inaccurate representation of the likely *in vivo* situation.

Several *in vitro* studies (Reijnen *et al.*, 1992, 1993) which used promoter constructs excluding both the C/EBP/DBP site (region -220 to -202) and the putative HNF4 binding site (region +20 to +45) resulted in a lower promoter activity than studies which incorporated both sites into their reporter gene assays. This indicated the importance of these two binding sites in the regulation of the factor IX promoter and indicated that promoter activity could be modulated by altering putative binding sites on the factor IX promoter. One could then speculate whether the *in vitro* analysis of the factor IX promoter by reporter gene constructs analysis could provide an accurate means of assessing the *in vivo* situation.

The difference between results obtained from research groups could also be attributed to the different cell lines utilised in each *in vitro* experiment. Several reports

have shown that a lack of transcription factors may be inherent in a particular cell line. For example, the human hepatoma cell line, HepG2 was shown to contain low concentrations of the transcription factor C/EBP. The results shown in Chapter 3 (section 3.7.2) indicated that the Alexander cell line was deficient in the transcription factor HNF4 when the activity of both normal and mutant promoter constructs was tested with varying amounts of the HNF4 expression plasmid. As a consequence of the lowered levels of transcription factors present within the cell line, the extent to which the promoter was expressed was altered *i.e* a lower level of expression was observed, and the inhibitory effects created by the promoter mutations may have also been masked. Therefore, an optimised transfection system was established in order to maintain optimal promoter expression.

The severity of haemophilia B prior to puberty, may result in part from both the location of the mutation within the factor IX promoter and the nucleotide substitutions that occur as a result. For example, the two mutations that occur within the -6 region of the factor IX promoter result in different severities of haemophilia B which is reflected by the different levels of factor IX protein found within the plasma of patients. The reporter gene assays in Chapter 3.3.5.2 also illustrated the different levels of transcription obtained from promoter constructs containing these two mutations, and have supported studies by Vidaud *et al.* (1993) who have directly implicated the -6 region in the regulation of the factor IX promoter. The reporter gene assay results in Chapter 3.3.5.2 also indicated that a mutation resulting in a purine base may have a more deleterious effect on promoter expression than a mutation resulting in a pyrimidine base. Such discrepancies in promoter activity may result from the changes in hydrogen-binding sites located in the major groove of DNA that are involved in the recognition and binding of DNA (Vidaud *et al.*, 1993).

The observation that neither of the Haemophilia B Leyden mutations at the -20 and -26 region of the promoter completely eliminated HNF4 binding in the current studies, suggested that remnant HNF4 binding could be required for the androgen-mediated response after puberty (Reijnen *et al.*, 1993). However, since the HNF4 transcription factor could transactivate the mutant (-6 G to C) factor IX promoter to a similar order of magnitude as the wild-type promoter, it was likely that the HNF4

transcription factor was capable of binding elsewhere on the promoter. The current investigation into the possible binding of the HNF4 transcription factor to the putative HNF4 site (+20 to +45), revealed that a mutation present within the +26 region of the promoter was unable to alter the level of factor IX transcription when compared to the wild-type promoter. This finding suggested that another HNF4 site may exist elsewhere on the factor IX promoter. Saturation mutagenesis of the factor IX promoter may assist in the discovery of other HNF4 sites present within the promoter. In order to establish whether the inability of the +26 G to C mutation to downregulate transcription was affected by either the level of endogenous transcription factors present within the cell line, or a weak mutation that was unable to disable the HNF4 binding site, further investigation by footprinting methods or mobility shift assays would be required.

Both *in vitro* and mobility shift assays using Cos 1 cell extracts have confirmed that the +5 to -15 region of the factor IX promoter is not an HNF4 binding site, thus casting doubt on studies carried out by Naka and Brownlee (1996). The ability of the HNF4 transcription factor to transactivate the factor IX promoter containing a -6 G to C mutation suggested that this site did not interfere with HNF4 binding.

These studies have emphasised the importance of establishing an optimised system with the Alexander cell line, which could reflect the likely situation that occurs *in vivo*.

4.2 FUTURE DIRECTIONS

4.2.1 Cultured cells: a feasible option?

The utilisation of mammalian cell lines in the study of the factor IX promoter can be both beneficial as well as disadvantageous. Although the *in vitro* situation enables a comparison to be made between the relative effects of promoter mutations and transcription activation, the problems that can occur with the cell line can alter the overall activity of the promoter and thus result in an inaccurate representation of the *in vivo* situation. In addition, the *in vitro* conditions utilised for each experiment can profoundly influence the outcome in transcription (Tjian and Maniatis, 1994).

One of the main limitations with using the mammalian cultured cell line is the inability to create or mimic the exact environmental conditions that occur within the normal liver cell. Although the transcriptional rates that occur within the hepatocyte are significantly higher than the levels observed within the human cultured cell (Poli *et al.*, 1990), the hepatocyte also undergoes a series of developmental changes which cannot be illustrated within the cultured cell line. As a result of the developmental changes that occur, the concentrations and availability of transcription factors within the hepatocyte are also altered. Therefore any difference in the concentrations of transcription factors present within a child and an adult resulting in the altered developmental regulation of the factor IX promoter cannot be analysed within the cultured cell line. The possible exclusion of other regulatory elements important to factor IX transcription may also occur with the *in vitro* studies.

The inability to determine the levels of transcription factors required by the cultured cell line to promote normal regulatory mechanisms within the factor IX promoter may also lead to further complications and possible misunderstandings when analysing and interpreting results. For example, the depletion of certain transcription factors within a cell line may mask or inhibit the effects of promoter mutations within the cell. On the other hand, the over-expression of a transcription factor within the cell line by incorporating various plasmids into reporter gene assays, may subsequently alter the normal regulatory mechanisms of the factor IX promoter therefore creating an inaccurate representation of the transcription factor *in vitro* (Franklin, 1995).

Future studies of the human factor IX promoter could be analysed using cell-free transcription systems or tissue extracts that can transcribe tissue-specific genes. These systems would enable the transcriptional machinery of the factor IX promoter to be analysed as a whole and would not require the supplementation of transcription factors important for efficient transcription. An alternative approach to this research may involve the use of primary hepatocytes, which represent the natural site of factor IX synthesis (Kay *et al.*, 1993). The endogenous transcripts produced from the primary hepatocytes represent the most physiologically relevant system, and could serve as an important system in which to analyse the roles of the transcription factors involved in the regulation of the factor IX promoter.

4.3 RECOVERY OF HAEMOPHILIA B LEYDEN: THE POSSIBLE MECHANISMS INVOLVED

The complex nature surrounding haemophilia B and the molecular mechanisms underlying the post-pubertal recovery from the disorder still remains enigmatic. The altered developmental expression that occurs with the onset of puberty suggests that the transcription regulation of the factor IX promoter must undergo substantial changes during this stage of development. The developmental timing of the recovery implicates the possible involvement of steroid hormones in factor IX regulation.

There has been considerable debate over the timing of the post-pubertal recovery of Haemophilia B Leyden. The role of the androgen receptor in the post-pubertal recovery has been of particular interest to research groups. To date, no one has reported strong transactivational evidence of the wild-type promoter when expressed with an androgen-receptor plasmid (Picketts *et al.*, 1993, 1994). Therefore the role of the androgen-receptor may be to mediate the action of other transcription factors that are capable of transactivating the factor IX promoter in the presence of testosterone. For example, an increase in the concentrations of transcription factors present within the hepatocyte may occur with the onset of puberty. An increase in transcription factors within the cell could therefore lead to a transactivation of the promoter to such an extent that any inhibitory effect created by a promoter mutation could be compensated for.

Based on the current *in vitro* studies, it is possible that the levels of the transcription factors C/EBP and HNF4 present within the cell prior to puberty may be substandard and as a consequence would be unable to enhance the activity of the factor IX promoter that was carrying a mutation at this stage of development. With the onset of puberty, the levels of transcription factor could increase dramatically with the surge of testosterone and the promoter could be transactivated to a level that would produce adequate amounts of factor IX protein in the plasma (~30 to 60 % of normal clotting activities).

A testosterone-dependant increase in the levels of transcription factors present within the hepatocyte would also explain the inability of factor IX levels to increase

with a mutation in the -26 region of the promoter. Testosterone is bound by androgen receptors (AR) present within the cell cytoplasm which subsequently interact with the androgen receptor element (ARE) located in the promoter region of the factor IX gene. One could theorise that the binding of the AR to the ARE may serve as a 'switch' to which the cell responds, by increasing the levels of transcription factor within the cell. The disruption of the ARE by the -26 mutation could subsequently hinder the binding of testosterone-AR complex to the ARE and, as a consequence, prevent the signal from being transmitted to increase the levels of transcription factor within the hepatocyte. The levels of transcription factor present within the hepatocyte would remain suboptimal.

The *in vitro* studies presented in Chapter 3 support the importance of the HNF4 and C/EBP transcription factors in the regulation of the factor IX promoter by their ability to transactivate both the normal and mutant promoter (region -220 to +45) constructs. The ability of the mutant promoter constructs to be transactivated to a level higher than the wild-type in the presence of both the HNF4 transcription factor and the C/EBP transcription factor adds further support to the above hypothesis. Further investigation using nuclear extracts may provide additional information on the various transcription factors present within the hepatocyte and how they developmentally regulate the factor IX promoter.

The presence of a second promoter within the factor IX gene has also been implicated by several research groups in the recovery of haemophilia B (Reitsma *et al.*, 1988; Crossley and Brownlee, 1990; Hirosawa *et al.*, 1990; Viduad *et al.*, 1993). Although a second promoter has not yet been discovered, it is possible that a second promoter might become activated if the main factor IX promoter is damaged due to a mutation. The activation of the second promoter may only occur with the onset of puberty and subsequently require the binding of the testosterone-AR complex to the ARE. A mutation within the -26 region of the factor IX promoter would therefore prohibit the activation of the second promoter.

The potential of protein species within the hepatocyte to dimerise with each other and other factors within the cell provides an unlimited scope for gene regulation to

occur. The compatibility of the leucine zippers and dimerisation domains between various protein species such as the C/EBP family and DBP transcription factor, indicates that dimerisation may in fact be a common mechanism for gene regulation due to the combined action of homodimeric and heterodimeric interactions with the factor IX promoter (Poli *et al.*, 1990). To date, the *in vitro* analysis of potential homo- or heterodimeric compounds within the cultured cell line has been limited. The C/EBP family have been implicated in the regulation of the factor IX promoter by either heterodimerising with other factors such as the D-site binding protein and IL-6 as well as being capable of dimerising with family members (Scott *et al.*, 1992). The *in vitro* studies presented here have illustrated the transactivational roles of the two C/EBP isoforms, C/EBPα and C/EBPδ with the factor IX promoter. The differential activities observed with all three isoforms may have been created by distinct interactions with other proteins (Osada *et al.*, 1996). Unfortunately, transient transfection analysis does not enable the detection of dimerisation between multiple partners suggesting that other methods of analysis may be required for future research. The potential of C/EBP isoforms to heterodimerise would suggest that they may be involved in a developmental role within the hepatocyte.

Further investigations into the ability of the C/EBP family members and other transcription factors associated with the factor IX promoter to heterodimerise *in vitro* may assist in elucidating the molecular mechanisms involved in the post-pubertal recovery of haemophilia B.

4.4 RESEARCH INTO THE REGULATION OF THE FACTOR IX PROMOTER

The specificity and efficiency of factor IX transcription depends upon a complex interaction of cell-specific and other general factors within the hepatocyte. The studies presented here have highlighted the importance of transcription factors and their roles in the transcriptional regulation of the factor IX promoter. In particular the *in vitro* studies have shown the transactivational capabilities of both C/EBP and HNF4 transcription factors on factor IX transcription. Furthermore these studies have suggested that the

factor IX promoter may be responsive to IL-6 thus implicating the possible involvement of the factor IX protein in the acute-phase response.

The studies presented here have assisted in the examination of the molecular mechanisms involved in the regulation of the factor IX promoter and may contribute in the elucidation of the mechanisms responsible for haemophilia B.

References

Aggler, P.M., White, S.G., Glendenning, M.B., Page, E.W., Bates, G (1952). Plasma thromboplastin component (PTC) deficiency: A new disease resembling hemophilia. *Proc Soc Exp Biol Med* **79**: 692-694.

Akira S., Isshiki H., Sugita T., Tanabe O., Kinoshita S., Nishio Y., Nakajima T., Hirano T., Kishimoto T (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J* **9 (6)**: 1897-1906.

Anson, D.S., Choo, K.H., Rees, D.J.G., Giannelli, F., Gould, K., Huddleston, J.A., and Brownlee, G.G (1984). The gene structure of human antihaemophilic factor IX. *EMBO J* **3**: 1053-1059.

Anson, D.S., Austen, D.E.G., and Brownlee, G.G (1985). Expression of active clotting factor IX from recombinant DNA clones in mammalian cells. *Nature* **315**: 683-685.

Aran, A., Cassuto, H., and Reshef, L (1995). Co-operation between Transcription Factors Regulates Liver Development. *Biol Neonate* **67**: 387-396.

Armenteno, D., Thompson, A.R., Darlington, G and Woo, S.L.C (1990). Expression of human factor IX in rabbit hepatocytes by retrovirus-mediated gene transfer: Potential for gene therapy of hemophilia B. *Proc Natl Acad Sci USA* **87**: 6141-6145.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G., and Struhl, K (1989). *Current Protocols in Molecular Molecular Biology. Volume I.* Greene Publishing Associates and Wiley Intersciences N.Y.

Babiss, L.E., Herbst, R.S., Bennett, A.L., and Darnell, J.E (1987). Factors that interact with the rat albumin promoter are present both in hepatocytes and other cell types. *Genes Dev* **1**: 256-267.

Balsover, S., Hyams, J., Jones, S., Shepard, G.A., White, H.A (1997). *From genes to cells*. Whiley-Liss, Inc., Third Avenue, N.Y.

Biggs, R., Douglas, A.S., Macfarlane, R.G., Davie, J.V., Pitney, W.R., Mersky, C., O'Brien, J (1952). Christmas disease: A condition previously mistaken for hemophilia. *Brit Med J* **2**: 1378-1382.

Brownlee, G.G (1987). The molecular pathology of haemophilia B. *Biochem Soc Trans* **15**: 1-8.

Brownlee, G.G (1995). Prospects for gene therapy of haemophilia A and B. *Brit Med Bull* **51 (1)**: 91-105.

Busby, S., Kumar, A., Joseph, M., Halfpap, L., Insley, M., Berkner, K., Kurachi, K., and Woodbury, R (1985). Expression of active factor IX in transfected cells. *Nature* **316**: 271-273.

Cao, Z., Umek, R.M., and McKnight, S.L (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* **5**:1538-1552.

Chen, X., Liu, W., Ambrosino, C., Ruocco, M., Poli, V., Romani, L., Quinto, I., Barbieri, S., Holimes, K., Venuta, S., and Scala, G (1997). Impaired generation of bone marrow β-lymphocytes in mice deficient in C/EBPβ. *Blood* **90 (1)**: 156-164.

Citarella F., Felici A., Brouwer M., Wagstaff J., Fantoni A., and Erik Hack, C (1997). Interleukin-6 downregulates factor XII production by human hepatoma cell line (HepG2). *Blood* **90 (4)**: 1501-1507.

Coyle, T.E., Spicer, T., Michalovic, D., and Poiesz, B.J (1994). Moderate Hemophilia B Leyden: Identification by Polymerase Chain Reaction, Sequencing, and Oligomer Restriction. *Am J Hematol* **46**: 234-240.

Crossley, M., and Brownlee, G.G (1990). Disruption of a C/EBP binding site in the factor IX promoter is associated with Haemophilia B. *Nature* **345**: 444-446.

Crossley, M., Winship, P.R., Austen, D.E.G., Rizza, C.R., and Brownlee, G.G (1990). A less severe form of Haemophilia B Leyden. *Nucleic Acids Res* **18**: 4633.

Crossley, M., Ludwig, M., Stowell, K.M., De Vos, P., Olek, K., and Brownlee, G.G (1992). Recovery from Haemophilia B Leyden: an androgen-responsive element in the factor IX promoter. *Science* **257**: 377-379.

De la Salle, H., Altenburger, W., Elkaim, R., Dott, K., Dieterlé, A., Drillien, R., Cazenave, J-P., Tolstoshev, P., and Lecocq, J-P (1985). Active γ-carboxylated human factor IX expressed using recombinant DNA techniques. *Nature* **316**: 268-270.

Franklin, I.K (1995). Characterisation of mutations at position -5 and -6 of the human factor IX gene. *M.Sc (Biochemistry) thesis*.

Giannelli, F., Green, P.M., Sommer, S.S., Lillicrap, D.P., Ludwig, M., Schwabb, R., Reitsma, P.H., Goossens, M., Yoshioka, A., and Brownlee, G.G (1994). Haemophilia B: a database of point mutations and short additions and deletions, fifth addition, 1994. *Nucleic Acids Res* **22**: 3534-3546.

Glover, D.M., and Hames, B.D (1995). *DNA Cloning 4: A practical approach*. Second edition. Oxford University Press Inc., N.Y.

Green, P.M., Montandon, A.J., Bentley, D.R., Ljung, R., Nilsson, I.M., and Gianelli F (1990). The incidence and distribution of CpG to TpG transitions in the coagulation factor IX gene. A fresh look at CpG mutational hotspots. *Nucleic Acids Res* **18 (11)**: 3227-3231.

Gerrard, A.J., Hudson, D.L., Brownlee, G.G and Watt, F.M (1993). Towards gene therapy for haemophilia B using primary keratinocytes. *Nature Genet* **3**: 180-183.

Herbomel, P., Bourachot, B., and Yaniv, M (1984). Two distinct enhancers with different cell specificities co-exist in the regulatory region of polyoma. *Cell* **39**: 654-661.

Hirosawa, S., Fahner, J.B., Salier, J.P., Wu, C.T., Lovrien, E.W., and Kurachi, K (1990). Structural and functional basis of the developmental regulation of human coagulation factor IX gene: factor IX Leyden. *Proc Natl Acad Sci USA* **87**: 4421-4425.

Hoeben, R.C., Fallaux, F.J., Cramer, S.J., van dem Wollenberg, D.J.M., van Ormondt, H., Briet., and van der Eb, A.J (1995). Expression of the blood clotting factor VIII cDNA is repressed by a transcriptional silencer located in its coding region. *Blood* **85 (9)**: 2447-2454.

Hoffbrand, A.V., and Pettit, J.E (1993). *Essential Haematology. Third Edition.* Blackwell Scientific Publications, London.

Holmes, D.S., and Quigley, M (1981). A rapid boiling method for the preparation of bacterial plasmids. *Anal Biochem* **114**:193-197.

Jones, N (1990). Transcriptional regulation by dimerisation: two sides to an incestuous relationship. *Cell* **61**: 9-11.

Kay, M.A., Rothenburg, S., Landen, C.N., Bellinger, D.A., Leland F., Toman, C., Finegold, M., Thompson A.R., Read, M.S., Brinkhous, K.M, and Woo, S.L.C (1993). *In vivo* gene therapy of hemophilia B: Sustained partial correction in factor IX-deficient dogs. *Science* **262**: 117-119.

Kay, M.A., Landen, C.N., Rothenburg, S.R., Taylor, L.A., Leland, F., Wiele, S., Fang, B., Bellinger, D., Finegold, M., Thompson, A.R., Read, M., Brinkhous, K.M., and Woo, S.L.C (1994). *In vivo* hepatic gene therapy: Complete albeit transient correction of factor IX deficiency in hemophilia B dogs. *Proc Natl Acad Sci USA* **91**: 2353-2357.

Kim, H.C., Smith, C., Matts, L., Eisele, J., and Saidi, P (1993). Continuous infusion of factor IX in a patient undergoing surgical procedure. *Blood* **82 (10)**:154a.

Kingdon, H.S., Brauker, J.H., Johnson, R.C., Carr-Brendel, V.E., Lozier, J.N., and High, K.A (1993). An immunoisolation device which facilitates cell-based prophylactic therapy for hemophilia. *Blood 82* **(10)**: 447a.

Kritis, A.A., Argyrokastritis, A., Moschonas, N.K., Power, S., Katrakili, N., Zannis, V.I., Cereghini, S., and Talianidis, I (1996). Isolation and characterisation of a third isoform of human hepatocyte nuclear factor 4. *Gene* **173**: 275-280.

Kurachi, K., Kurachi, S., Furukawa, M., and Yao, S-N (1993). Biology of factor IX. *Blood Coag Fib* **4**: 953-974.

Kurachi, S., Furukawa, M., Salier, J-P., Wu, C-T., Wilson, E.J., French, F.S., and Kurachi, K (1994). Regulatory mechanism of human factor IX gene: protein binding at the Leyden-specific region. *Biochem* **33**: 1580-1591.

Kurachi, K., and Kurachi, S (1995). Regulatory mechanisms of the factor IX gene. *Thromb Haem* **73 (3)**: 333-339.

Ladias, J.A.A., Hadzopoulou-Cladaras, M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V., and Cladaras, C (1993). Transcriptional regulation of human apolipoprotein genes apo B, apo CIII and apo AII by members of the steroid hormone receptor superfamily HNF4, ARP-1, EAR-2, and EAR-3. *J Biol Chem* **267**: 15849-15860.

Lamb, P.T., and McKnight, S.L (1991). Diversity and specificity in transcriptional regulation: the benefits of heterotypic dimerization. *TIBS* **19**: 417-422.

Lai, E., and Darnell, J.E (1991). Transcriptional control in hepatocytes: a window on development. *TIBS* **16**: 427-429.

Majello B., Arcone R., Toniatti C., and Ciliberto G (1990). Constitutive and IL-6 induced nuclear factors that interact with the human C-reactive protein promoter. *EMBO J* **9 (2)**: 457-465.

Naka, H., and Brownlee, G.G (1996). Transcriptional regulation of the human factor IX promoter by the orphan receptor superfamily factors, HNF4, ARP1 and COUP/EAR3. *Br J Haematol* **92**: 231-240.

Nishiyori, A., Tashiro, H., Kimura, A., Akagi, K., Yamamura, K., Mori, M., and Takiguchi, M (1994). Determination of tissue specificity of the enhancer by combinatorial operation of tissue-enriched transcription factors. *J Biol Chem* **269 (2)**: 1323-1331.

Osada, S., Yamamoto, H., Nishihara, T., and Imagawa, M (1996). DNA binding specificity of the CCAAT/Enhancer-binding protein transcription factor family. *J Biol Chem* **271 (7)**: 3891-3896.

Palmer, T.D., Thompson, A.R., and Miller, A.D (1989). Production of human factor IX in animals by genetically modified skin fibroblasts: Potential therapy for hemophilia B. *Blood* **73 (2)**: 438-445.

Pang, C.P., Crossley, M., Kent, G., and Brownlee, G.G (1990). Comparative sequence analysis of mammalian factor IX promoter. *Nucleic Acids Res* **18**: 6731-6732.

Pavletich, N.P., and Pabo, C.O (1991). Zinc finger-DNA recognition: crystal structure of a Zif 268-DNA complex at 2.1 Å. *Science* **252**: 809-817.

Picketts, D.J., D'Souza, S., Bridge, P.J., Lillicrap, D (1992). An A to T transversion at position -5 of the factor IX promoter results in haemophilia B. *Genomics* **12**: 161-163.

Picketts, D.J., Lillicrap, D.P., and Mueller, C.R (1993). Synergy between transcription factors DBP and C/EBP compensates for a Haemophilia B Leyden factor mutation. *Nature Genet* **3**: 175-179.

Picketts, D.J., Mueller, C.R., and Lillicrap, D (1994). Transcriptional control of the factor IX gene: analysis of five cis-acting elements and the deleterious effects of naturally occurring Haemophilia B Leyden mutations. *Blood* **84 (9)**: 2992-3000.

Poli, V., Mancini, F.P., and Cortese, R (1990). IL-6 DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* **63**: 643-652.

Purrello, M., Alhadeff, B., Espsito, D., Szabo, P., Rocchi, M., Truett, M., Masiarz, F., and Siniscalco, M (1985). The human genes for haemophilia A and B flank the X-chromosome fragile site at Xq27.3. *EMBO J* **4 (3)**: 725-729.

Reijnen, M.J., Sladek, F.M., Bertina, R.M., and Reitsma, P.H (1992). Disruption of a binding site for hepatocyte nuclear factor 4 results in Haemophilia B Leyden. *Proc Natl Acad Sci USA* **89**: 6300-6303.

Reijnen, M.J., Peerlinck, M., Maasdam, D., Bertina, R.M., and Reitsma, P.H (1993). Haemophilia B Leyden: substitution of thymine for guanine at position -21 results in disruption of a hepatocyte nuclear 4 binding site in the factor IX promoter. *Blood* **82**: 151-158.

Reijnen, M.J., Maasdam, D., Bertina, R.M., and Reitsma, P.H (1994). Haemophilia B Leyden: the effect of mutations at position +13 on the liver-specific transcription of the factor IX gene. *Blood Coag Fibrin* **5**: 341-348.

Reitsma, P.H., Bertina, R.M., Ploos van Amstel, J.K., Riemens, A., Briet, E (1988). The putative factor IX gene promoter in Haemophilia B Leyden. *Blood* **72**: 1074-1076.

Roberts, H.R (1993). Molecular Biology of Hemophilia B. *Thromb Haem* **70 (1)**: 1-9.

Roeder, R.G (1991). The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. *TIBS* **16**: 402-407.

Royle, G., Van De Water, N.S., Berry, E., Ockelford, P.A., Browett, P.J (1991). Haemophilia B Leyden arising *de novo* by point mutations in the putative factor IX promoter. *Br J Haematol* **77**: 191-194.

Rubin, E., and Forber, J.L (1988). *Pathology.* J.B Lippincott and Co., Philadelphia.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual. Second Edition, Book 3.* Cold Spring Harbour Laboratory Press.

Sanger, F., Nicklen, S. and Coulen, A.R (1977). DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 1677-1678.

Scott, L.M., Civin, C.I., Rorth, P., Friedman, A.D (1992). A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. *Blood* **80**: 1725-1735.

Singer, S (1978). *Human Genetics. An introduction to the Principles of Heredity.* W.H. Freeman and Co., San Francisco.

Solomon, E.P., Schmidt, R.R., and Adragna, P.J (1990). *Human Anatomy and Physiology. Second Edition.* Saunders College Publications., Florida.

Steel, D.M., and Whitehead, A.S (1994). The major acute phase reactants; C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol today* **15 (2)**: 81-88.

Thompson, A.R (1986). Structure, function, and molecular defects of factor IX. *Blood* **67**: 565-572.

Thompson, A.R (1991). Molecular biology of the hemophilias. *Prog Hemostasis Thromb* **10**: 175-214.

Tjian, R., and Maniatis, T (1994). Transcriptional activation: a complex puzzle with few easy pieces. *Cell* **77**: 5-8

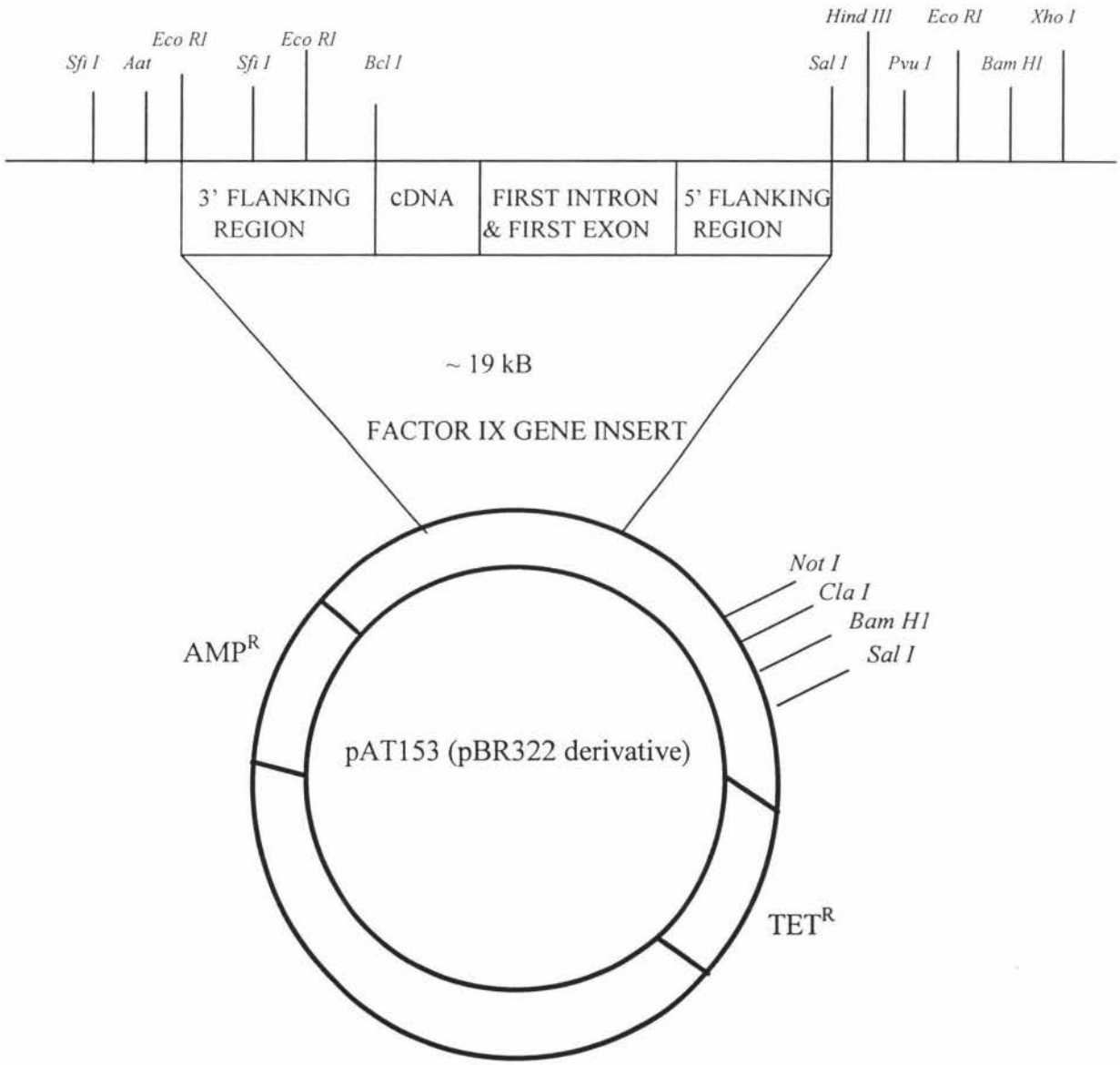
Vidaud, D., Tartary, M., Costa, J.M., Bahnak, B.R., Suzanna, G.S., Fressinaud, E., Gazengel. C,, Meyer, D., Goossens, M., Lavergne, J.M., Viduad, M (1993). Nucleotide substitution at the -6 position in the promoter region of the factor IX gene results in different severity of Haemophilia B Leyden: consequences for genetic counselling. *Hum Genetics* **91**: 241-244.

Watson, J.D., Gilman, M., Witkowski, J., and Zoller, M (1992). *Recombinant DNA. Second Edition.* Scientific American Books, W.H Freeman and Company, New York, U.S.A.

Yao, S.N., and Kurachi, K (1992). Expression of human factor IX in mice after injection of genetically modified myoblasts. *Proc Natl Acad Sci USA* **89**: 3357-3361.

Appendix 1

Map of pTG3954



Appendix 2

DNA sequences used in PCR:

Amplification of the factor IX promoter sequence (-220 to +60) from pTG3954.

1916 GTG CTG CCA CAG TAA ATG TA
1917 TGA TGA GGC CTG GTG ATT CT

DNA sequences used in sequencing:

PCR primers for sequencing inserts into pGL2 Basic

2825 CTC ATC AAT GTA TCT TAT GGT ACT G
2826 TGG CGT CTT CCA TTT TAC CAA CAG

Sequencing primers for sequencing inserts into pGL2 Basic

2827 CTT ATG GTA CTG TAA CTG AG
2828 TTA CCA ACA GTA CCG GAA TG

PCR primers for sequencing inserts in pGL2 Basic biotinylated 5'

2858 CTC ATC AAT GTA TCT TAT GGT ACT G
2859 TGG CGT CTT CCA TTT TAC CAA CAG

DNA sequences used in EMSA:

The human factor IX promoter from region -40 to -9 (-26 G to C mutation)

1579 AGC TTA TAC AGC TCA GCT TCT ACT TTG GTA CAA CTA
1580 AGC TTA GTT GTA CCA AAG TAG AAG CTG AGC TGT ATA

The α1-antitrypsin gene promoter LF-A1/HNF4 site

1589 GAC TCA GAT CCC AGC CAG TGG ACT TAG CCC CTG TTT
1590 AAA CAG GGG CTA AGT CCA CTG GCT GGG ATC TGA GTC

The human factor IX promoter from -23 to +6 (-6 G to C mutation)

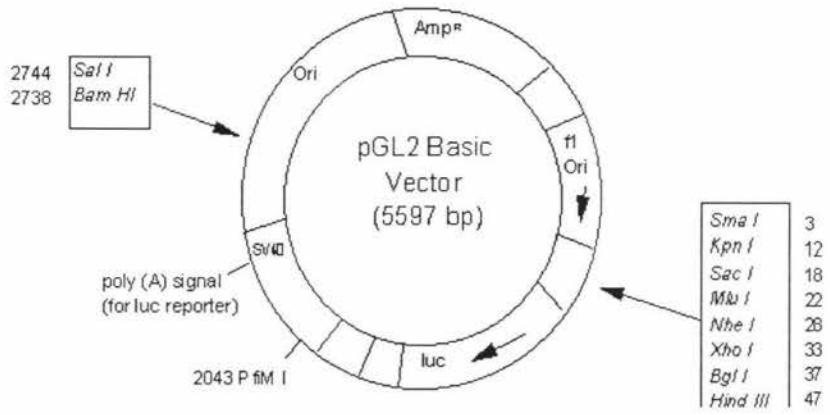
1648 AGC TTT GGT ACA ACT AAT CCA CCT TAC CAC TA
1649 AGC TTA GTG GTA AGG TGG ATT AGT TGT ACC AA

The human factor IX promoter from region -14 to +6

1712 AGC TTA ACT AAT CGA CCT TAC CAC TA
1713 AGC TTA GTG GTA AGG TCG ATT AGT TA

Appendix 3

A Map of the vector pGL2 Basic



Vector Map Notes:

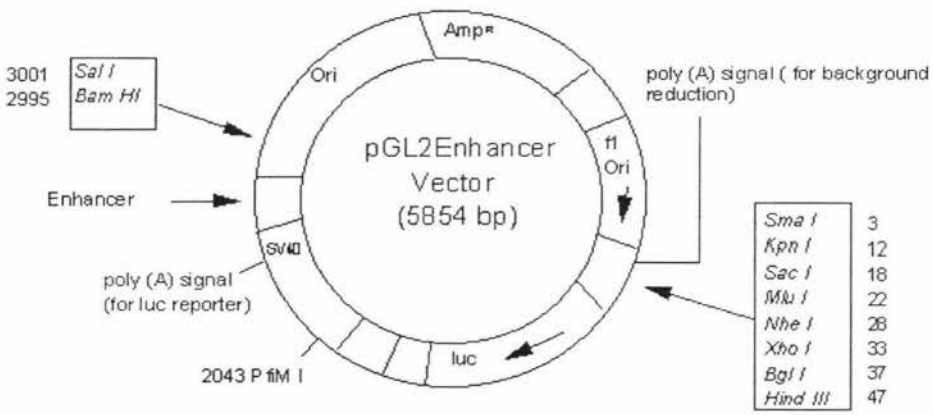
1. Sequence reference points
   a. SV40 regions:

Promoter	(none)
Enhancer	(none)
Intron	1968-2033
3' untranslated region	1892-2743

   b. Luciferase gene (luc) 76-1725
   c. β-lactamase (Amp^R) 4674-3817
   d. f1 originColE1-derived plasmid replication origin 3052

Appendix 4

Map of the Vector pGL2 Enhancer

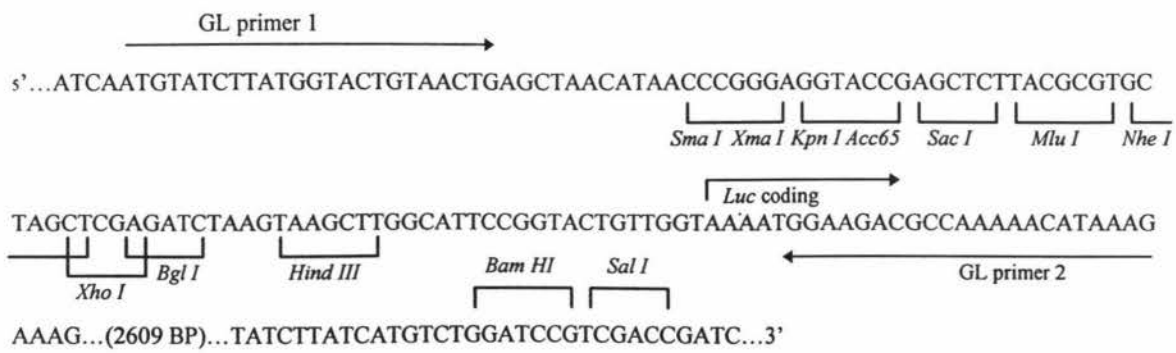


Vector Map Notes:

1. Sequence reference points

a. SV40 regions	
Promoter	(none)
Enhancer	2739-2995
Intron	1968-2033
3' untranslated region	1892-2742
b. Luciferase gene (luc)	76-1725
c. β-lactamase (Amp^R)	4931-4074
d. f1 origin	5063-5518
e. ColE1-derived plasmid replication origin	3309

Multiple Cloning Region for pGL2 Vectors



Appendix 5

Map of pMT2-HNF4