1071

PROMOTOR MUTATIONS IN A PATIENT WITH HAEMOPHILIA B LEYDEN.
P.H. Reitsma, A.M. Riemens, R.M. Bertina, E. Briët. Haemostasis and Thrombosis Research Unit, Leiden University Hospital, Leiden, The Netherlands.

Haemophilia B Leyden is characterized by low levels of factor IX antigen and activity before the age of 15, whereas after puberty factor IX levels rise at a rate of about 4-5% per year. To date such a genetic variant of factor IX synthesis has been reported in two (probably related) Dutch families, in a Greek and in an American family of Armenian descent. Laboratory and clinical investigations indicate that the factor IX protein is normal but that the regulation of factor IX synthesis has come under the control of the steroid hormone testosterone. We have started to investigate the factor IX gene in a patient from a Dutch family in an attempt to explain the aberrant regulation.

Southern blotting revealed no gross deletions or insertions in the factor IX gene. Therefore the promotor region of the factor IX gene was cloned and subjected to a detailed restriction enzyme analysis. This also did not indicate that significant DNA deletions or insertions had occurred. Subsequently we established the nucleotide sequence of the DNA surrounding the first exon which encompassed about 600 basepairs of the promotor region. Two deviations from previously published sequence data were recorded. Firstly, an A T change was noted in the presumed "tata" box region 20 basepairs upstream from the start site of mRNA synthesis. Secondly, at position -423 a T C change was found which lies 13 basepairs upstream from a potential alternative "tata" box.

The point mutation at position -20 might well explain the failure of gene expression during the prepuberal stage of the disease. Whether the same point mutation also leads to the testosterone effects or that a second sequence variation is a prerequisite for this phenomenon remains to be established from studies on the promotor region in representatives of the Greek and American families. Eventually the introduction of chaemeric genes, containing the various promotor regions, into testosterone responsive cells should delineate the promotor sequences responsible for the variations in factor IX gene expression.

1072

GENE DELETIONS IN THE FACTOR IX LOCUS.
R.J. Matthews (1), D.S. Anson (2), I.R. Peake (1), A.L. Bloom (1). Department of Haematology, University of Wales College of Medicine, Cardiff, Wales UK(1). Sir William Dunn School of Pathology, Oxford England UK(2).

Previous studies have indicated that the majority of haemophilia B patients who produce anti-factor IX inhibitors (antibodies) have some kind of deletion of the factor IX gene. We have analysed DNA from nine haemophilia B inhibitor patients using the Southern blotting method and hybridisation with (i) factor IX cDNA and intragenomic probes (ii) probes originating from flanking sequences up to 60kb 5' and 170kb 3' to the factor IX gene that have been isolated by gene walking experiments (D.S.Anson and G.G.Brownlee, unpublished observations).

Two patients who are brothers (haemophilia B (Chicago I)) were shown to have a presumably identical complex rearrangement of the factor IX gene involving two separate deletions. The first deletion is approx. 5.0kb and removes exon e. The second deletion is between 9 and 29kb and removes exons g and h but leaves exon f intact. An abnormal TaqI fragment at one end of the deletions junctions acted as a marker for the inheritance of haemophilia B in the patients' family. Furthermore, an abnormal 11kb BglII fragment (detected with an intragenomic probe containing exon f) in DNA from both patients and their mother acted as a marker for the presence of both deletions. Since the patients' grandmother only showed the normal 12kb Bgl II fragment then both deletions appear to have arisen at the same time. We believe that haemophilia B (Chicago 1) is the first observation of a natural gene rearrangement involving two separate deletions within the same gene.

Patient haemophilia B (Jersey 1) was revealed to have a deletion of at least 170kb including the entire factor IX gene and $>$ 60kb of 5' flanking sequence. The 3' breakpoint of this deletion was mapped to between 80 and 140kb 3' to the factor IX gene. One further patient, haemophilia B (Boston I) was shown to have a deletion of $>$ 230kb including the factor IX gene, $>$ 60kb of 5' flanking sequence and $>$140kb of 3' flanking sequence. Five other inhibitor patients had a structurally intact gene as detected by this method.

Although all nine haemophilia B inhibitor patients studied did not have a detectable plasma factor IX only in four of them is this absence due to a large deletion of the factor IX gene.

1073

MOLECULAR BASIS OF HEMOPHILIA B: IDENTIFICATION OF THE DEFECT IN FACTOR IX VANCOUVER.
V.A. Geddes, G.V. Louie, G.D. Brayer and R.T.A. MacGillivray. Department of Biochemistry, University of British Columbia, Vancouver, Canada.

Factor IX Vancouver (fIX-V) is the cause of a moderate form of hemophilia B. An individual presenting with this disorder had 2.6% of normal procoagulant activity in his plasma but had 62% of the normal factor IX antigen level. Specific antibodies showed that fIX-V contains epitopes for both the heavy and light chains of factor IXa. To identify the defect involved, DNA was isolated from the lymphocytes of the male hemophiliac. Southern blot analysis using a full-length factor IX cDNA as a hybridization probe showed no gross differences between the fIX-V gene and the normal factor IX gene. The DNA from the hemophiliac was then partially digested with Sau3A and the resulting fragments (10-20kbp in size) were ligated into the BamHI site of λEMBL3. The DNA was then packaged into phage particles *in vitro*, and the recombinant phage were screened with the factor IX cDNA as a probe. Eight phage were isolated that contained overlapping DNA covering the complete gene for fIX-V. DNA sequence analysis of the protein-encoding regions, the intron/exon junctions and 5'- and 3'-flanking sequences revealed a single nucleotide change from the normal factor IX gene. The codon for amino acid 397 was changed from ATA (Ile) to ACA (Thr). This mutation is in the catalytic domain of factor IXa and is novel amongst those hemophilia B mutations reported to date. Based on the known three dimensional structures of the pancreatic serine proteases, trypsin, elastase and chymotrypsin, models have been constructed for the structures of the catalytic domains of both the normal and Thr-397 mutant of factor IXa. These results suggest that the Thr-397 mutation may alter the conformation of the substrate binding region in the active site of factor IXa Vancouver through the formation of a hydrogen bond between the hydroxyl group of the Thr-397 side chain and the main chain carbonyl group of Trp-385. The postulated conformational change would lead to reduced binding affinity for the factor IXa substrate resulting in a reduction in the catalytic activity of fIXa-Vancouver.

Supported in part by grants from the Medical Research Council of Canada (to GDB and RTAM).

1074

MOLECULAR ANALYSIS OF COAGULATION FACTOR VIII AND IX GENES BY DNA PROBES.
C. de la Salle (1), M.J. Baas (1), L. Grunebaum (1), R. Gialeraki (2), T. Mandalaki (2) and J.-P. Cazenave (1). INSERM U.311, Service d'Hémostase et de Thrombose, Centre Régional de Transfusion Sanguine, Strasbourg, France (1) and Regional Blood Transfusion Center, Athens, Greece (2).

About 250 individuals belonging to 44 families with hemophilia A or B were studied in our laboratory. The detection of carriers was first established by pedigree analysis of each family and coagulation and immunological assays of factor VIII or IX. The availability of specific probes for the molecular study of these two genes makes possible a diagnosis with certainty in the case of informative families. 25 families of hemophilia A were studied. For each person, blood was collected into EDTA and leucocyte DNA was extracted, digested by restriction endonucleases, electrophoresed in 0.9 % agarose gels and transferred to nitrocellulose filters by Southern blotting. Two probes were used for the analysis of factor VIII gene. The St 14 probe (J.L. Mandel) located on the q28 region of the X chromosome and closely linked to the gene, determines a restriction fragment length polymorphism (RFLP) when the DNA is digested by the enzyme Taq_I. The p114-12 genomic probe (Genentech) corresponding to the exons 17 and 18 of the factor VIII gene, reveals a RFLP in the DNA digested by the enzyme Bcl_I. 19 families of hemophilia B were studied. A total factor IX cDNA probe was used for the screening of potential deletions in the case of hemophiliacs with circulating antibodies. A genomic probe containing the exons II, III and IV of factor IX was used to detect the Taq_I RFLP. For the study of factor VIII gene, the extragenic probe St 14 gives a very high percentage of informativity (about 90 %) but recombination can occur between the probe and the gene. The p 114-12 probe, which is used to confirm the results given by the St 14 probe, gives about 20 % informativity. In our study, we were able to diagnose carrier state with certainty in 92 % of the families. For hemophilia B, the genomic probe gives about 40 % informativity. A large deletion of the region of the factor IX gene has been found in one family and remains to be mapped. In conclusion, carrier detection and prenatal diagnosis can be established with certainty by molecular studies in most cases of hemophilia A using the St 14 probe, with a 5 % risk of recombination when the Bcl_I RFLP cannot confirm. This diagnosis is possible in about 40 % of the cases of hemophilia B.