the slowing of the recovery following Cl^- injection in the presence of furosemide suggest a furosemide-sensitive net outward transport of Cl^-. In GC, the E_{IPSP} was fairly independent of whether 1*M* KCl or 0.6*M* K_2SO_4 was present in the recording electrode. Yet, the results of Cl^- injections indicated that the IPSP was dependent on Cl^- and that, because of rapid recovery, an outward pump must be present in GC. However, a furosemide-sensitive outward pump seems difficult to reconcile with the usually depolarizing IPSP of GC. Because the depolarizing IPSP is diminished in the presence of furosemide, GC must have an additional inward pump mechanism. Yet only an outwardly directed Cl^- transport mechanism has been described in mammalian central neurons (*1*). However, there is an inward pump sensitive to ammonium (*15*) or furosemide in frog motoneurons (*16*) and rat sympathetic neurons (*17*). The furosemide-induced shifts of the apparent E_{IPSP} and of the GABA reversal potential observed in this study suggest that the internal Cl^- activity of GC and CA3 cells is regulated by inward and outward Cl^- transport processes. A homogeneous distribution of inward and outward Cl^- pumps in GC and a dendritic and somatic localization of these pumps in CA3 cells (*18*) accounts for both the changes in E_{IPSP} and the observed shifts in the multiphasic GABA responses.

REFERENCES AND NOTES

1. H. D. Lux, *Science* **173**, 555 (1971); R. Llinas, R. Baker, W. Precht, *J. Neurophysiol.* **37**, 522 (1974).
2. R. A. Deisz and H. D. Lux, *J. Physiol. (London)* **326**, 123 (1982); C. C. Aickin, R. A. Deisz, H. D. Lux, *ibid.* **329**, 319 (1982).
3. B. E. Alger and R. A. Nicoll, *Nature (London)* **281**, 315 (1979); R. H. Thalmann, E. J. Peck, G. F. Ayala, *Neurosci. Lett.* **21**, 319 (1981).
4. P. Andersen, R. Dingledine, L. Gjerstad, I. A. Langmoen, A. Mosfeldt-Laursen, *J. Physiol. (London)* **305**, 279 (1980).
5. J. Cousin and R. Motais, *ibid.* **256**, 61 (1976); P. C. Brazy and R. B. Gunn, *J. Gen. Physiol.* **68**, 583 (1976).
6. U. Misgeld, J. M. Sarvey, M. R. Klee, *Exp. Brain Res.* **37**, 217 (1979). The saline, which was equilibrated with 5% CO_2 and 95% O_2 contained 124 m*M* NaCl, 5 m*M* KCl, 1.3 m*M* $MgSO_4$, 1.25 m*M* KH_2PO_4, 26 m*M* $NaHCO_3$, 2.5 m*M* $CaCl_2$, and 10 m*M* glucose at *p*H 7.4 and 36°C. The intracellular electrodes (60 to 100 megohms) contained either 1*M* KCl or 0.6*M* K_2SO_4. Bipolar electrodes were positioned at the mossy fibers to activate GC antidromically and at the perforant path to activate GC orthodromically. CA3 cells were stimulated antidromically by the Schaffer collaterals and orthodromically by the mossy fibers.
7. Amplitudes of both hyperpolarizing and depolarizing IPSP's were measured 25 msec after the stimulation at the peak amplitude of the hyperpolarizing IPSP's.
8. J. C. Eccles, R. A. Nicoll, T. Oshima, F. J. Rubia, *Proc. R. Soc. Lond. Ser. B.* **198**, 345 (1977).
9. U. Misgeld, M. R. Klee, M. L. Zeise, in *Physiology and Pharmacology of Epileptogenic Phenomena*, M. R. Klee, H. D. Lux, E. J. Speckmann, Eds. (Raven, New York, 1982), pp. 131–139.
10. GABA was applied by pressure from micropipettes (approximately 5 μm) containing 10 m*M* GABA in saline.
11. Furosemide was provided by Hoechst (Federal Republic of Germany) or purchased from Sigma.
12. J. R. Hotson, D. A. Prince, P. A. Schwartzkroin, *J. Neurophysiol.* **42**, 889 (1979). The rectification ratio was calculated by dividing the input resistance at −55 mV by that at −70 mV ($n = 6$).
13. N. R. Newberry and R. A. Nicoll, *J. Physiol. (London)* **348**, 239 (1984).
14. B. E. Alger and R. A. Nicoll, *ibid.* **328**, 125 (1982).
15. There are contradictory reports concerning ammonium sensitivity of outward transport in hippocampal CA1 neurons. Chloride transport in rat CA1 neurons was found to be insensitive to ammonium [B. E. Alger and R. A. Nicoll, *J. Neurophysiol.* **49**, 1381 (1983)], but was ammonium-sensitive in guinea pig CA1 neurons because ammonium induced a positive shift in the E_{IPSP} after intracellular iontophoresis of Cl^- [J. R. Hotson and D. A. Prince, *J. Neurophysiol.* **43**, 409 (1980)]. This corresponds to our own observations. Whereas 0.5 m*M* furosemide was sufficient to induce a positive shift in E_{IPSP} of CA3 neurons after intracellular Cl^- iontophoresis, only 2 m*M* furosemide induced a clear shift in E_{IPSP} of CA3 neurons at normal intracellular Cl^- concentration.
16. C. P. Bührle and U. Sonnhof, *Neuroscience* **14**, 581 (1985).
17. K. Ballanyi and P. Grafe, *J. Physiol. (London)*, in press.
18. GABA can hyperpolarize as well as depolarize CA1 pyramidal cell dendrites [B. E. Alger and R. A. Nicoll, *ibid.* **328**, 125 (1982); R. K. S. Wong and D. J. Watkins, *J. Neurophysiol.* **48**, 938 (1982)]. The explanation that these effects are mediated by two different receptors is contradicted by our conclusion indicating two spatially separated inward and outward Cl^- pumps. The two-receptor hypothesis depends on the assumption that GABA applied to the dendrites does not reach somatic receptors.

19. We thank B. Weber for technical assistance. Supported by a grant to the SFB 220 of the Deutsche Forschungsgemeinschaft.

4 November 1985; accepted 25 February 1986

Identification of a Missense Mutation in the Factor VIII Gene of a Mild Hemophiliac

JANE GITSCHIER,* WILLIAM I. WOOD, MARC A. SHUMAN, RICHARD M. LAWN

DNA probes derived from the cloned factor VIII gene can be used to detect mutations in the factor VIII gene of hemophiliacs. DNA hybridization analysis led to the identification of two contrasting point mutations in the same codon. In a severe hemophiliac with no detectable factor VIII activity, the normal arginine codon (number 2307) is converted to a stop codon, while in a mild hemophiliac with 10 percent of normal activity, this same codon is converted to glutamine.

THE GENETIC DISEASE HEMOPHILIA A (classic hemophilia) is caused by a defect in the blood coagulation protein, factor VIII. From the cloning of the human factor VIII gene (*1–4*), the sequence of the 2332–amino acid protein has been determined. This sequence data provides the basis for understanding the mechanism of action of factor VIII, and the cloned gene has been instrumental in elucidating the molecular basis of hemophilia. We have previously reported the use of cloned DNA probes to demonstrate examples of both deletion and point mutations in the factor VIII gene of several patients with severe hemophilia (*5*). All of the point mutations in these patients resulted in stop codons (nonsense mutations). We now describe an amino acid substitution due to a missense mutation that occurs in a mild hemophiliac. This amino acid change is in the same codon in which a nonsense mutation led to severe disease.

In our initial survey of hemophilia DNA samples (*5*), the enzyme Taq I proved to be particularly useful for detecting mutations in the factor VIII gene. The recognition sequence for Taq I (TCGA) contains the dinucleotide CpG, the most common site of methylation in human DNA and a possible site of increased mutation due to deamination of cytosine leading to C to T transitions (*6*). In two surveys of hemophilia DNA's for changes in the Taq I sites within the factor VIII gene, the expected mutation was identified in three of the seven Taq I sites occurring in the protein-coding regions (*5, 7*). One example we have described is the case of a severe hemophiliac, H22, having no detectable factor VIII activity, in which a Taq I site normally present in the coding region of exon 26 (the 3′-most exon) is missing (*5*). Cloning and sequencing of the H22 DNA in this region demonstrated the expected C to T change in the CpG dinucleotide of the Taq I site. This mutation converts a CGA (arginine) codon to a TGA (stop) codon 26 amino acids before the normal chain termination site (Fig. 1).

The same Taq I site is also missing in the DNA of another patient, H104, who is a mild hemophiliac (9 percent factor VIII activity). To account for the phenotypic difference between these two individuals, we

J. Gitschier, W. I. Wood, R. M. Lawn, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080.
M. A. Shuman, Hematology Section, Cancer Research Institute, University of California, San Francisco, CA 94143.

*Present address: Howard Hughes Medical Institute, University of California, San Francisco, CA 94143.

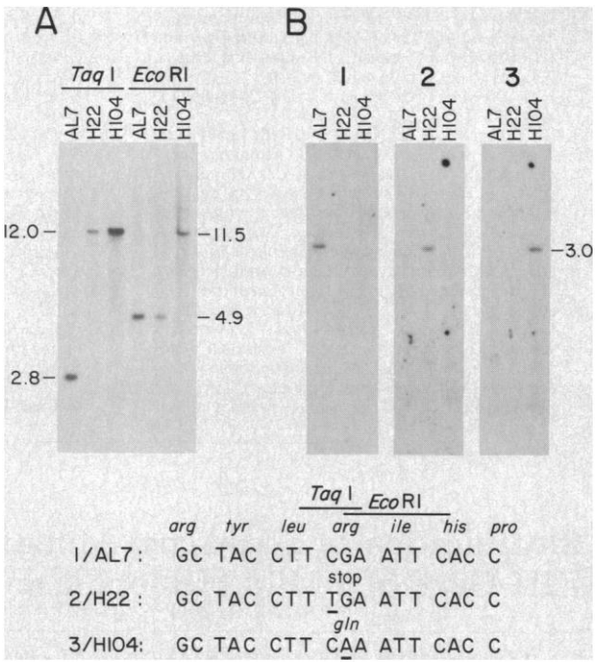

Fig. 1. Analysis of the mutant factor VIII gene in patient H104. (A) A Southern blot of AL7 (nonhemophilic), H22, and H104 DNA's (5 μg per lane), digested with either Taq I or Eco RI, was hybridized (*5*) to a 1.9-kb Eco RI complementary DNA fragment that contains exon 26 (*2*). The normal 2.8-kb Taq I fragment in the AL7 DNA (*5*) is shifted to 12 kb in H22 and H104 DNA, whereas the normal 4.9-kb Eco RI fragment in AL7 DNA, also present in H22 DNA, is shifted to 11.5 kb in H104 (the 5′-adjacent Eco RI fragment in normal DNA is 6.6 kb). (B) Ten micrograms of Rsa I–digested DNA from the AL7 cell line and from patients H22, and H104 were separated by electrophoresis on each of three 1 percent agarose gels. The gels were hybridized individually to one of three 18-base oligonucleotides (bottom). The normal (AL7) and mutant (H22 and H104) amino acid sequences are indicated. The oligomers were end-labeled with ^{32}P. Gels were dried (*8*), hybridized, and washed at 55°C in 3*M* TMACl as described (*9*). Films were exposed 2 to 4 days at −70°C with one intensifying screen. (The same results were obtained with a trio of 22-base oligomers with a central mismatch washed at 61°C in TMACl, but with somewhat less discrimination in hybridization.)

postulated that the mutation in the Taq I site in H104 occurred by a C to T transition in the reverse complement strand of DNA. This would produce a G to A change in the coding strand and replace an arginine codon with a glutamine codon (Fig. 1). A Southern blot of DNA digested with Eco RI was consistent with this proposal. The partially overlapping Eco RI site in the H104 DNA is lost along with the Taq I site, as predicted by this nucleotide substitution (Fig. 1).

To determine the sequence of H104 DNA in this region, a series of three 18-base oligomers was synthesized, differing at a single internal position: probe 1 has the sequence of normal (nonhemophilic) DNA, prepared from the cell line AL7 (*2*), probe 2 has the H22 DNA sequence, and probe 3 has the proposed sequence for H104 (Fig. 1, bottom). These oligomers were end-labeled with ^{32}P and hybridized individually to three identical gel panels (*8*), each containing DNA from AL7, H22, and H104. The gels were washed in 3*M* tetramethylammonium chloride (TMACl) at 55°C. TMACl eliminates the preferential stability of GC versus AT base pairs and is an improvement upon the use of standard saline citrate (SSC) since the wash conditions need not be determined empirically for each sequence (*9*). The blots were washed at 55°C, a temperature 2° to 3°C below the dissociation temperature for an 18-base oligonucleotide (*9*). Under these conditions AL7 DNA hybridizes only to probe 1, H22 DNA only to probe 2, and H104 only to probe 3 (Fig. 1B). Therefore, at conditions which are sufficiently stringent to discriminate a single-base mismatch in the control DNA samples, H104 hybridizes with probe 3 exclusively, thus demonstrating that the sequence in H104 is as predicted. High stringency washes with TMACl eliminate the extraneous binding often associated with oligonucleotide hybridization to genomic DNA in SSC.

The missense mutation in H104 leads to the substitution of a positively charged arginine by a neutral glutamine residue. We cannot be certain that the missense mutation is responsible for the hemophilia in patient H104. Other base changes in the 186,000 bp of the factor VIII gene or at other loci might also affect the protein. However, it is likely for several reasons that the reported base change would result in the mild hemophilia phenotype. First, this base change has not been seen in 220 other human X chromosomes (80 normal and 140 hemophilic) that we have examined; hence it is not a common polymorphism associated with normal or hemophilic individuals. The mother of H104, a known carrier of hemophilia, is heterozygous for the mutation. Second, the affected amino acid residue may be important for the integrity of the protein. The arginine residue is located in the 80-kD fragment of the protein retained in the thrombin-activated state (*10*), and it is conserved in the two copies of the homologous C domains that comprise the carboxyl terminal portion of factor VIII (*1*). The substitution of an uncharged for a positively charged amino acid could have a destabilizing or inactivating effect on the molecule. Finally, all of the deletion and chain termination (nonsense) point mutations that have thus far been described for factor VIII occur in patients with severe hemophilia. It is reasonable that some missense mutations would lead to mild disease.

The mild hemophilia in patient H104 is due to reduced levels of circulating factor VIII rather than inactivation of the protein itself. The patient has about 6 percent of the normal amount of factor VIII antigen. In this case, as in about 90 percent of all cases of mild hemophilia, a diminished level of factor VIII antigen coincides with the level of clotting activity. Although some of these cases would be expected to arise from mutations affecting rates of synthesis or secretion, subtle changes in the protein could also affect activation or stability. However, the altered amino acid in this case does not occur in a position known to be cleaved by thrombin, factor X, or activated protein C during the process of activation and subsequent inactivation of factor VIII (*10*). Factor VIII was partially purified from patient H104 with the use of a monoclonal antibody specific for the 80-kD fragment (*2*). It was found to clot factor VIII–deficient plasma and respond to thrombin activation in a normal fashion (*11*). Hence the missense mutation in the factor VIII gene of this individual might produce a clotting factor that functions rather normally, but is relatively unstable and more quickly degraded. In this regard it resembles several unstable hemoglobin variants of otherwise normal activity that are caused by amino acid substitutions (*12*).

REFERENCES AND NOTES

1. J. Gitschier *et al.*, *Nature (London)* **312**, 326 (1984).
2. W. I. Wood *et al.*, *ibid.*, p. 330.
3. G. A. Vehar *et al.*, *ibid.*, p. 337.
4. J. J. Toole *et al.*, *ibid.*, p. 342.
5. J. Gitschier *et al.*, *ibid.* **315**, 427 (1985).
6. D. Barker, M. Schafer, R. White, *Cell* **36**, 131 (1984).
7. S. E. Antonarakis *et al.*, *N. Engl. J. Med.* **313**, 842 (1985).
8. B. J. Conner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 278 (1983).
9. W. I. Wood, J. Gitschier, L. A. Lasky, R. M. Lawn, *ibid.* **82**, 1585 (1985).
10. D. Eaton, H. Rodriguez, G. Vehar, *Biochemistry* **25**, 505 (1986).
11. D. H. Eaton, Genentech, Inc., personal communication.
12. H. F. Bunn, B. E. Forget, H. M. Ranney, *Human Hemoglobins* (Saunders, Philadelphia, 1977).

13. We thank Dr. Daniel Eaton of Genentech, Inc., for performing and communicating analysis of factor VIII protein, Dr. Leon Hoyer of the University of Connecticut for measuring factor VIII antigen, and Mark Vasser and Peter Ng of Genentech for synthesis of the oligonucleotides. This work was supported by Genentech, Inc.

16 December 1985; accepted 14 February 1986