Antithrombin III Utah: Proline-407 to Leucine Mutation in a Highly Conserved Region near the Inhibitor Reactive Site†

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ABSTRACT: A dysfunctional antithrombin III (ATIII) gene encoding a qualitatively and quantitatively abnormal anticoagulant molecule is responsible for hereditary thrombosis in a Utah kindred [Bock et al. (1985) *Am. J. Hum. Genet. 37*, 32–41]. Nucleotide sequencing of the entire protein-encoding portion of the cloned ATIII-Utah gene revealed a C to T transitional mutation which converts proline-407 to leucine. Proline-407 is located 14 amino acids C-terminal to the reactive site arginine of ATIII in a core region of the molecule that has been highly conserved during evolution of the serine protease inhibitor (serpin) gene family. The location of this proline in the crystal structure of the homologous serpin α1-antitrypsin suggests that the leucine substitution in ATIII-Utah may interfere with correct folding of the mutant gene product, leading to its rapid turnover and the low antithrombin levels observed in patient plasmas. The Pro-407 to Leu mutation does not interfere with binding of antithrombin III to heparin. Patient antithrombin III, isolated by affinity chromatography on heparin–Sepharose, was reacted with purified thrombin. ATIII encoded by the patient's normal gene formed protease–inhibitor complexes with thrombin, whereas the product of the ATIII-Utah gene did not. The Pro-407 to Leu mutation destroys a restriction site for the enzyme *Stu*I, permitting rapid diagnosis of affected members of the Utah kindred by Southern blotting of genomic DNA.

Antithrombin III (ATIII)[1] is a plasma protease inhibitor that inhibits thrombin and plays an important role in maintaining the fluidity of blood. ATIII functions as an anticoagulant by forming extremely stable, proteolytically inactive complexes with thrombin and other protease targets (XIIa, IXa, Xa, XIa) (Rosenberg & Damus, 1973; Travis & Salvesen, 1983). The rate of complex formation is increased over 3 orders of magnitude by the sulfated mucopolysaccharide heparin (Rosenberg & Damus, 1973). Separate parts of the ATIII molecule are thought to interact with thrombin and heparin.

ATIII is a member of the serine protease inhibitor (serpin) gene family (Carrell, 1984). This family of homologous proteins includes known plasma protease inhibitors such as ATIII, α1-antitrypsin, C1 inhibitor, α1-antichymotrypsin, heparin cofactor II, α2-antiplasmin, protein C inhibitor, and plasminogen activator inhibitor, as well as proteins with no known inhibitor function such as ovalbumin, angiotensinogen, barley protein Z, and thyroxine binding globulin.

Those serpins that do function as protease inhibitors employ a common mechanism. A region called the reactive site is located on a loop protruding from the molecule (Loebermann et al., 1984; Carrell & Owen, 1985) and contains an amino acid sequence that is an ideal substrate for the target protease of the serpin. Stoichiometric 1:1 enzyme–inhibitor complexes form rapidly between serpins and their target proteases. The proteases are inactive in these enzyme–inhibitor complexes, which have a negligible rate of dissociation and are stable during SDS–polyacrylamide electrophoresis under reducing conditions.

Antithrombin III deficiency causes thrombosis by reducing the anticoagulant activity of plasma. Many families with ATIII deficiency and hereditary thrombosis have been described [see Thaler and Lechner (1981) for review]. Inheritance of this condition is autosomal dominant, and affected individuals (who are *heterozygous* for the trait) may experience recurrent episodes of deep vein thrombosis and pulmonary embolism during adult life.

Immunological, biochemical, functional, and DNA-level studies have established that the molecular bases of ATIII deficiency are heterogeneous. A classification system based on the relative amounts of ATIII antigen and its functional activity in plasma has been proposed (Sas et al., 1980). Type I deficiencies ("classical") are caused by mutations that interrupt ATIII biosynthesis at the DNA, RNA, or protein levels. In affected individuals from type I families, plasma ATIII antigen and activity levels are both reduced to approximately 50% of normal. Type II deficiencies ("functional") are those in which an abnormal anticoagulant molecule is produced from the mutant allele; in these cases antigen levels exceed the amount of functional activity present.

The subject of the present report is a large Utah kindred in which 13 members in 3 generations have had ATIII deficiency. This family was originally described as a type I classical deficiency (Cosgriff et al., 1983) since ATIII antigen and activity levels are both reduced to approximately 50% of normal. However, subsequent immunoblotting studies revealed the presence of very small amounts of a variant ATIII molecule in the plasma of Utah patients (Bock et al., 1985), indicating that this family actually belongs in the type II functional deficiency category. The present report shows that the protein product of the ATIII-Utah gene does not form protease–in-

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[1] Abbreviations: ATIII, antithrombin III; serpin, serine protease inhibitor; bp, base pair; SDS, sodium dodecyl sulfate; RFLP, restriction fragment length polymorphism.

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hibitor complexes with thrombin but does have normal heparin binding affinity. We also used molecular cloning to isolate the antithrombin III allele which encodes ATIII-Utah and identified a C to T mutation in the second position of the codon for proline residue 407. This mutation is located in a highly conserved region of the inhibitor, very near to the reactive site.

Experimental Procedures

Genomic Cloning. Two two-allele RFLPs have been identified in the human ATIII locus. The alleles of the *Pst*I sequence polymorphism (Prochownik et al., 1983a) are designated + and −, while those of the length polymorphism (Bock & Levitan, 1983) are called F and S. A previous study (Bock et al., 1985) established tight linkage of the mutant gene and the +,S DNA polymorphism haplotype in the Utah kindred (LOD = 3.35 at $\Theta = 0.0$). Patient II-9 is heterozygous for both ATIII RFLPs (−,F/+,S haplotype). Genomic DNA was prepared from peripheral blood cells of patient II-9, partially digested with *Sau*3A, and then size fractionated by centrifugation through a 10–40% sucrose gradient. An EMBL-3 (Frischauf et al., 1983) phage library was constructed from the 15–20-kb fraction and screened with ^{32}P-labeled ATIII cDNA probe (Bock et al., 1982). DNA polymorphism haplotypes of ATIII gene containing recombinants were determined in order to identify clones originating from the Utah (+,S) and normal (−,F) alleles.

Sequencing. The human ATIII gene consists of seven exons interrupted by six introns [Prochownik et al. (1985) and this paper]. The exons and flanking intron regions of an ATIII-Utah allele (λB) were sequenced, as was exon 6 of the normal allele from II-9 (λD). Plasmid 10-2B (generously provided by E. Prochownik) was the DNA source for mapping and sequencing exons 3A and 3B of the normal ATIII gene. The ATIII allele contained in 10-2B originated from an *Eco*RI partial human genomic DNA library (Fritsch et al., 1980) and is the same allele whose exon–intron structure was described in Prochownik et al. (1985). All nucleotide sequence data were obtained from dideoxy chain-termination reactions (Sanger et al., 1977) on plasmid subclones of the human DNAs and analyzed by using computer programs developed in the Biomathematics Computation Laboratory at the University of California, San Francisco.

Southern Blot Analysis. Southern blots (Southern, 1975) were prepared from 3-μg samples of genomic DNA which had been digested with *Stu*I and hybridized to ^{32}P-nick-translated fragments of ATIII cDNA.

Heparin–Sepharose Chromatography. An initial heparin–Sepharose chromatography experiment was conducted to determine if ATIII-Utah binds heparin. Later, heparin–Sepharose chromatography was used to purify ATIII for complex formation experiments.

Plasma was prepared by centrifugation of whole blood that had been anticoagulated with citrate phosphate dextrose (approximately 450 mL of blood was collected into 63 mL of CPD solution containing 1.66 g of sodium citrate, 1.61 g of dextrose, 206 mg of citric acid, and 140 mg of monobasic sodium phosphate). Protein concentration was determined by using a Bio-Rad protein assay kit. Aliquots (100 mL) of plasma which had been stored at −70 °C were thawed, diluted with an equal volume of 50 mM Tris-HCl, pH 7.5, 40 mM sodium citrate, and 0.02% sodium azide, and cleared by centrifugation at 3500g for 10 min. Diluted plasma samples were passed through a 1.5 × 17 cm heparin–Sepharose (Pharmacia) column at 25 mL/h. Following extensive washing with 0.25 M NaCl and 50 mM Tris-HCl, pH 7.5, bound material was eluted with a linear gradient composed of 60 mL of the above and 60 mL of 3 M NaCl and 50 mM Tris-HCl, pH 7.5. Aliquots of each fraction were examined by electrophoresis of reduced and denatured samples on Laemmli gels (Laemmli, 1970) which were silver stained (Merril et al., 1981) or immunoblotted (Burnette, 1981).

Complex Formation. A heparin–Sepharose fraction of Utah patient plasma containing 160 μg/mL ATIII (calculated by assuming $E^{1\%}$ per cm at 280 nm = 6.5) in 0.9 M NaCl and 10 mM Tris-HCl, pH 7.5, was activated with heparin (15 units/mL) for 5 min at 37 °C. Aliquots (25 μL) of human thrombin (containing the amounts of thrombin indicated in the Figure 5 legend in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.2 mM benzamidine) were added to 12.5-μL aliquots of the heparin-activated ATIII. Following an additional 2.5-min incubation at 37 °C, an equal volume of 6% SDS, 7.5% β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, and 30% glycerol was added and the samples were boiled for 3 min prior to electrophoresis on Laemmli gels and silver staining.

Results

As a result of this work on the ATIII-Utah gene, a small intron was discovered in what had been previously reported as exon 3 of the normal human antithrombin III gene (Prochownik et al., 1985). Nucleotide sequence analysis of the Utah allele (Figure 1A) revealed the presence of a 1-kb intron between codons 176 and 177 of what had been reported as exon 3 (codons 105–222). When it was reexamined by restriction mapping and DNA sequencing (Figure 1B), the previously analyzed normal allele was found to contain the same intron. Thus, the human ATIII gene consists of seven exons separated by six introns. Exon 3A includes amino acid residues 105–176 and is separated from exon 3B, which includes residues 177–222, by a kilobase of intervening sequence. The donor and acceptor splice sites for this intron conform to the "GT-AG rule" (Breathnach & Chanbon, 1981) (see Figure 1B for nucleotide sequence). The genes of other serpin family members for which the structures have been determined do not contain a homologously placed intron, suggesting that this intervening sequence evolved after divergence of antithrombin III from these other serpins. The revised structure of the human antithrombin III gene is presented in Figure 5.

In a previous linkage study utilizing ATIII gene restriction fragment length polymorphisms (RFLPs), the gene encoding ATIII-Utah was shown to reside on a chromosome of the +,S haplotype (Bock et al., 1985). This work also identified several affected members of the Utah kindred who are heterozygous for at least one of the ATIII RFLP markers. DNA from patient II-9 was selected for use in cloning experiments because this individual is heterozygous both at the 5′ length polymorphism (Bock & Levitan, 1983) and at the more 3′ sequence polymorphism (Prochownik et al., 1983a). A size-fractionated, *Sau*3A partial digest library was constructed in λ phage pEMBL-3. Six recombinants containing antithrombin III genes were identified and their ATIII DNA polymorphism haplotypes determined. One of the six clones contained the entire 15-kb ATIII locus of the mutant +,S allele. Comparison of Southern blots of phage and genomic DNA showed that no gross rearrangements of the ATIII gene had occurred during library construction and propagation of the DNA in phage.

The seven exons of the Utah gene and the 5′ and intervening sequence regions flanking them were sequenced (Figure 1). Table I summarizes sequence differences between the ATIII-Utah gene and the sequences of several normal anti-

A

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SEGMENT 1:
ctgcagacaa gtttctcctc agtcaggtat ttcctaacca agtttgaggg tatgaacata  60
ctctcctttt ccttttctat aaagctgagg agaagagtga gggagtgtgg gcaagagagg 120
tggctcaggc tttccctggg cctgattgaa ctttaaaact tctctactaa ttaaacaaca 180
ctgggctcta cactttgctt aaccctggga actggtcatc agcctttgac ctcagttccc 240
cctcctgacc agctctctgc cccaccctgt cctctggaac ctctgcgaga tttagaggaa 300
agaaccagtt ttcaggcgga ttgcctcaga tcacactatc tccacttgcc cagccctgtg 360
          ▲mRNa start
gaagattagc ggccatgtat tccaatgtga taggaactgt aacctctgga aaaaggtaag 420
                                                exon 1▲ivs 1
aggggtgagc t
SEGMENT 2:
tgtacttggt tcaaaggatt tagcctttct cttggccaca ccaggtgggc tggaatcctc  60
tgctttactg gggcaaccct gtggtgggca gtggggctag gggttgcagc ctagcttaac 120
ttggcatttt gtctccttgc aggaaggttt atcttttgtc cttgctgctc attggcttct 180
              ivs 1▲exon 2
gggactgcgt gacctgtcac gggagccctg tggacatctg cacagccaag ccgcgggaca 240
ttcccatgaa tcccatgtgc atttaccgct ccccggagaa gaaggcaact gaggatgagg 300
gctcagaaca gaagatcccg gaggccacca accggcgtgt ctgggaactg tccaaggcca 360
attcccgctt tgctaccact ttctatcagc acctggcaga ttccaagaat gacaatgata 420
acatttttct gtcacccctg agtatctcca cggcttttgc tatgaccaag ctgggtgcct 480
gtaatgacac cctccagcaa ctgatggagg tacgaccaaa ggtcttctgc ccagccacct 540
                  exon 2▲ivs 2
tgttaggaac acctttgggc cttccatagg cccaagtcca atgattcctc aaccaacact 600
gcag
SEGMENT 3a:
cttttatcct tttattcatc agaacacaag agttgagcat ttatgctgtc ccaggtactg  60
tgcttgaagg agttaacaac tgaggtggct attagtcaga gactgaccag catgtgctca 120
ccacccatgt taactaggca gcccaccaaa cccaccacca ttttttttg acttctatag 180
                                                        ivs 2
gtatttaagt ttgacaccat atctgagaaa acatctgatc agatccactt cttctttgcc 240
▲exon 3a
aaactgaact gccgactcta tcgaaaagcc aacaaatcct ccaagttagt atcagccaat 300
cgccttttg gagacaaatc ccttaccttc aatgagacct accaggacat cagtgagttg 360
gtatatggag ccaagctcca gccccctggac ttcaaggtga gttgcagatg ttacccctga 420
                    exon 3a▲ivs 3a
cctccgagtt cttcctctcc actcagagat tgaggaggtg gagaaacagc atccaaattc 480
acactgcttt gctgctgaag actgctggag ggctgactaa aagttagaac ccctgcaata 540
gttattctta cttgaaacct gagaatcaaa ggtatccatg cttggattgt actgactgcc 600
cagaaaacat gaattgaata atcaattctt cattccatcc acca
SEGMENT 3B:
tgctcttttt cttctgataa tatttattaa ctacaggaaa gattcatgga actatattag  60
atatgtaggc ttcccaattt gggttagagc aatggcttct taatcaaatg gtgggaaagg 120
acagagggat ggtgagaaaa ataaaatgct gcctgggaaa atggagaagc caattgaata 180
gcacaggtga gtaggtttat tttctgttct cctcaggaaa atgcagagca atccagagcg 240
                                ivs 3a▲exon 3B
gccatcaaca aatgggtgtc caataagacc gaaggccgaa tcaccgatgt cattccctcg 300
gaagccatca atgagctcac tgttctggtg ctggttaaca ccatttactt caaggtactc 360
                                                exon 3B▲ivs 3B
agaatggccc tggagagacc ccagggactt cctcttgctc ttcagcttac ccccttttt 420
tttaaatggc gagaccgaag ccctgagagg gcaaatggac tgccgaaagc tacacaggta 480
caggtcagca gggcaggtca atctattatt tatttattta tttatttttg acagagtctc 540
gctctgtcgc ccaggctgga gtgcagtggc gtgatctcgg ctcactg
SEGMENT 4:
tttttccgaa taattatata ttaatgtaac actataatat ggatatgtct gtgtcaataa  60
ctatcctcct atgaatgttt gtgttcttac tttgtgattc tcttccaggg cctgtggaag 120
                                                ivs 3B▲exon 4
tcaaagttca gccctgagaa cacaaggaag gaactgttct acaaggctga tggagagtcg 180
tgttcagcat ctatgatgta ccaggaaggc aagttccgtt atcggcgcgt ggctgaaggc 240
acccaggtgc ttgagttgcc cttcaaaggt gatgacatca ccatggtcct catcttgccc 300
aagcctgaga agagcctggc caaggtggag aaggaactca ccccagaggt gctgcaggag 360
tggctggatg aattggagga gatgatgctg gtggtccaca tgccccgctt ccgcattgag 420
gacggcttca gtttgaagga gcagctgcaa gacatgggcc ttgtcgatct gttcagccct 480
gaaaagtcca aactcccagg tttgtctagg aaggagtttc ctcccttctc caccccgcaag 540
                exon 4▲ivs 4
gtagtctgac caaaagtgga agagttggag aaagaataga aa
SEGMENT 5:
gaattcccat ctgtggattg aagccaactt tctcccatct cacaaagact tctccggtct  60
tcttccaggt attgttgcag aaggccgaga tgacctctat gtctcagatg cattccataa 120
  ivs 4▲exon 5
ggcatttctt gaggtgagta caccttcccc actctcttag ggtacagaaa ggagatgcat 180
              exon 5▲ivs 5
gaacagcagg aacagtggaa aaggcctgtt tccagtgtta aggcatgca
SEGMENT 6:
ctgcaggtaa atgaagaagg cagtgaagca gctgcaagta ccgctgttgt gattgctggc  60
ivs 5▲exon 6
cgttcgctaa accccaacag ggtgactttc aaggccaaca ggcttttcct ggtttttata 120
agagaagttc ctctgaacac tattatcttc atgggcagag tagccaaccc ttgtgttaag 180
taaaatgttc ttattctttg cacctcttcc tatttttggt ttgtgaacag aagtaaaaat 240
▲stop
aaatacaaac tacttccatc tcacattata aatggactct gcatttgaaa tgaagataag 300
gaaaggggaa acatgctatt gggg
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B

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SEGMENT 3a:
aactaggcag cccaccaaac ccaccaccat ttttttttga cttctatagg tatttaagtt  60
                                        ivs 2▲exon 3a
tgacaccata tctgagaaaa catctgatca gatccacttc ttctttgcca aactgaactg 120
ccgactctat cgaaaagcca acaaatcctc caagttagta tcagccaatc gccttttgg 180
agacaaatcc cttaccttca atgagaccta ccaggacatc agtgagttgg tatatggagc 240
caagctccag cccctggact tcaaggtgag ttgcagatgt taccccctgac ctccgagttc 300
                                exon 3a▲ivs 3a
ttcctctcca ctcagagatt gaggaggtgg agaaacagca tccaaattga cactgctttg 360
ctgctgaaga ctgctggagg gctgactaaa agttagaac
SEGMENT 3B:
ggacagaagg atggtgagaa aaataaaatg ctgcctggga aaatggagaa gccaattgaa  60
tagcacaggt gagtaggttt attttctgtt ctcctcagga aaatgcagag caatccagag 120
                                ivs 3a▲exon 3B
cggccatcaa caaatgggtg tccaataaga ccgaaggccg aatcaccgat gtcattccct 180
cggaagccat caatgagctc actgttctgg tgctggttaa caccatttac ttcaaggtac 240
                                                exon 3B▲ivs 3B
tcagaatggc cctggagaga ccccagggac ttcctcttgc tcttcagctt acccccttt 300
tttttaaatg gcgagaccga agccctgaga gaggcaaatg gactgccgaaa gctacacagg 360
tacaggtcag cagggcaggt caatctatta tttatttatt tatttatttt tgacagagtc 420
tcgctctgtc gcccaggctg gagtgcagtg gcgtgatctc ggctcactg
SEGMENT 6:
ctgcaggtaa atgaagaagg cagtgaagca gctgcaagta ccgctgttgt gattgctggc  60
ivs 5▲exon 6
cgttcgctaa accccaacag ggtgactttc aaggccaaca ggcttttcct ggtttttata 120
agagaagttc ctctgaacac tattatcttc atgggcagag tagccaaccc ttgtgttaag 180
taaaatgttc ttattctttg cacctcttcc tatttttggt ttgtgaacag aagtaaaaa
▲stop
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FIGURE 1: (A) Nucleotide sequence of ATIII-Utah gene. A phage library was generated from the genomic DNA of an individual who is heterozygous for ATIII deficiency (II-9; Bock et al., 1985) The ATIII gene restriction fragment length polymorphism haplotypes of antithrombin III gene containing recombinants were determined in order to differentiate clones originating from the mutant (+,S haplotype) and normal (−,F haplotype) alleles. "Segments" (consisting of exons and adjacent intron regions) of the mutant gene were sequenced. Differences between the sequences of the Utah allele and several different normal alleles are summarized in Table I. The underlined T residue in exon 6 causes the Pro-407 to Leu Utah mutation. (B) Nucleotide sequences of segments 3A, 3B, and 6 from normal ATIII genes. The sequences of segments 3A and 3B were determined from subclones of the normal ATIII allele described in Prochownik et al. (1985). The sequence of segment 6 was determined from the normal, −,F haplotype allele of Utah family member II-9.

Table I: Comparison of ATIII-Utah and Normal ATIII Gene Sequences

segment/ region[a]	DNA sequenced (no. of bases) ATIII-Utah	DNA sequenced (no. of bases) normal ATIII (ref)	sequence differences[k] position[a]	DNA source (ref)	sequence
1/5′	304	534[d,e]	121	Utah	T
				normal[e]	C
				normal[d]	T
			207	Utah	GGGAACTGGTCA
				normal[e]	AGGACCTGGTCAA
				normal[d]	GGGAACTGGTCA
			271	Utah	C
				normal[e]	T
				normal[d]	C
1/exon 1	110	110[d,e,f]	305	Utah	CC
				normal[e]	CCC
				normal[d]	CC
			337	Utah	TA
				normal[e]	TCA
				normal[d]	TA
			371	Utah	GGC
				normal[e]	CCG
				normal[d]	GGC
1/IVS 1	16	284[d,e,f]	427	Utah	GA
				normal[e,g]	CG
				normal[d]	GA
2/IVS 1	142	32[g]			
2/exon 2	367	367[h,f,i]	203	Utah	G
				normal[f]	A
				normal[h]	G
			222	Utah	A
				normal[i]	G
				normal[h,f]	A
			391	Utah	A
				normal[i]	G
				normal[h,f]	A
2/IVS2	95	33[g]			
3A/IVS 2	95	33[b,g]	177	Utah	AT
				normal[g]	GC
				normal[b]	AT
3A/exon 3A	216	216[b,h,i,f]	291	Utah	A
				normal[f]	G
				normal[b,h,i]	A
3A/IVS 3A	248	133[b]			
3B/IVS 3A	216	98[b]			
3B/exon 3B	138	138[b,h,i,f]	231	Utah	A
				normal[i]	G
				normal[b,h,f]	A
			240	Utah	G
				normal[f]	T
				normal[b,h,i]	G
			279	Utah	A
				normal[f]	T
				normal[b,h,i]	A
3B/IVS 3B	233	233[b,g]	365	Utah	T
				normal[g]	C
				normal[b]	T
			372	Utah	GGAGAGA
				normal[g]	GAGAG
				normal[b]	GGAGAGA
4/IVS 3B	108	29[g]			
4/exon 4	391	391[h,i,f,j]	204	Utah	G
				normal[f]	A
				normal[h,i]	G
			216	Utah	C
				normal[i]	T
				normal[h,f]	C
			327	Utah	G
				normal[f]	A
				normal[h,i,j]	G
			357	Utah	G
				normal[f,i]	A
				normal[h,i,j]	G
			396	Utah	C
				normal[h]	T
				normal[f,i,j]	C
4/IVS 4	83	37[g,j]	526	Utah	TTCTCC
				normal[g]	ACCCGC
				normal[j]	TTCTCC
5/IVS 4	68	19[g]			
5/exon 5	65	65[h,i,f]			
5/IVS 5	96	24[g]			

Table I (Continued)

segment/ region[a]	DNA sequenced (no. of bases) ATIII-Utah	normal ATIII (ref)	sequence differences[k] position[a]	DNA source (ref)	sequence
6/IVS 5	6	6[b,g]			
6/exon 6	318	216[b,h,i,f,j]	104	Utah	T
				normal[b,h,i,f,j]	C
			105	Utah	C
				normal[b,i,f,j]	C
				normal[h]	T[c]

[a] Segment identification numbers and position numbering as in Figure 1. [b] This paper. [c] Reanalysis of original clone indicates that this nucleotide is a C. [d] Bock & Levitan, 1983. [e] Prochownik & Orkin, 1984. [f] Chandra et al., 1983. [g] Prochownik et al., 1985. [h] Bock et al., 1982. [i] Prochownik et al., 1983b. [j] Jagd et al., 1985. [k] Of the thirteen differences between nucleotide sequences in the polypeptide coding regions, ten are silent third-position substitutions, while three are first- or second-position base changes that result in amino acid substitutions. Two of these amino acid substitutions affect normal alleles as well as the ATIII-Utah allele.

thrombin III alleles. Normal gene sequences were obtained from three independently isolated antithrombin III cDNAs (Bock et al., 1982; Prochownik et al., 1983b; Chandra et al., 1983) and segments of three independently isolated ATIII genes [Prochownik et al., 1985; Jagd et al., 1985; and the normal allele (−,F haplotype) of II-9]. Numerous conflicts were noted between the Utah gene and the normal ATIII alleles in the 304 bp of 5′ sequence, 1606 bp of exons, and 693 bp of intervening sequence compared. However, with one exception, each of these differences could be attributed to the presence of DNA sequence polymorphisms or sequencing errors. This conclusion was reached by comparing the Utah sequence to the sequences of several normal alleles and noting that although it was in conflict with particular allele(s), it was in agreement with other(s). In contrast, a sequence conflict in the second position of ATIII-Utah codon 407 could not be resolved on comparison with the sequences of five different normal alleles. Whereas a C is present at this position in the normal genes, a T is present in the Utah allele, causing substitution of a leucine for a proline.

In addition to causing an amino acid substitution at the protein level, the Utah mutation destroys a restriction site that is present in exon 6 of the normal gene. Figure 2A shows that the nucleotides encoding arginine-406 and proline-407 form a *Stu*I recognition site in the normal ATIII gene. This site is destroyed by the Utah mutation, and as a consequence, a new 6.6-kb fragment is observed on Southern blots of Utah patient DNAs prepared with *Stu*I (Figure 2B). DNAs from 10 normal, 7 affected, and 7 unaffected Utah family members, and from 13 affected members of 10 other ATIII deficiency kindreds, were examined on *Stu*I Southern blots. The 6.6-kb fragment was observed in all Utah patient samples, but not in other samples (data not shown).

Plasma from an ATIII-Utah patient was chromatographed on heparin–Sepharose in order to determine whether the Pro-407 to Leu mutation affects binding of the Utah protein to heparin. Aliquots (100 mL) of normal and Utah plasma containing equivalent amounts of total protein were applied to heparin–Sepharose columns (Figure 3). The ATIII peak (solid black bar) from Utah plasma is approximately half as large as the corresponding peak from an equal volume of normal plasma, and accordingly, Laemmli gel analysis revealed that approximately twice as much of the normal antithrombin III isoforms n and β (Peterson & Blackburn, 1985), which differ in their degree of glycosylation (Brennan et al., 1987), are present in normal plasma compared to Utah plasma. In addition to these normal ATIII species, Utah plasma contains a band (u) which (1) coelutes from heparin–Sepharose with the major ATIII isoform n, (2) has greater electrophoretic mobility than it, and (3) is present at greatly reduced concentration compared to the protein products of the normal allele (n and β). This band corresponds to the ATIII-Utah protein described in a previous genetic linkage and immunoblotting study (Bock et al., 1985). Altered electrophoretic mobility of ATIII-Utah relative to normal ATIII is apparently due to anomalous binding of SDS. Similar mobility shifts have

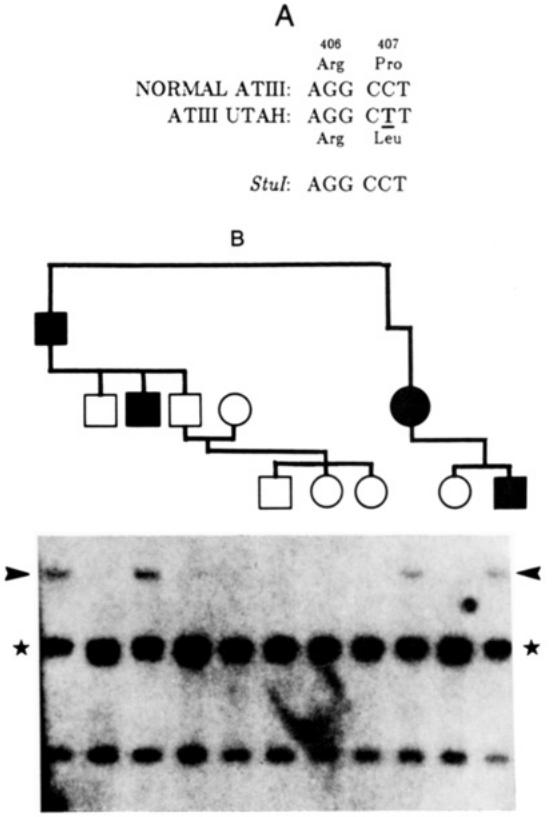

FIGURE 2: (A) Nucleotide sequence, amino acid sequence, and restriction site change associated with the ATIII-Utah mutation. Sequences at codons 406 and 407 are shown. The ATIII-Utah mutation substitutes a T for a C in the second position of codon 407, causing replacement of a proline with a leucine. A *Stu*I recognition site is present in the 406–407 position of the normal ATIII gene; the Utah allele is *Stu*I resistant. (B) Segregation of 6.6-kb *Stu*I fragment containing the Pro-407 to Leu mutation in the Utah family. A Southern blot was prepared with *Stu*I and hybridized to the 500 base pair *Pst*I fragment from the 3′ end of the human ATIII cDNA (Bock et al., 1982). Solid symbols indicate affected family members. The 6.6-kb Utah band is marked with arrowheads. Note that the 3.3-kb "band" marked with stars contains multiple hybridizing fragments, two of which contain exon 7 sequences and together form a 6.6-kb band in the Utah allele. The intensity of the 6.6-kb mutant band seems abnormally weak in the autoradiogram due to the presence of the multiple hybridizing fragments in the 3.3-kb "band".

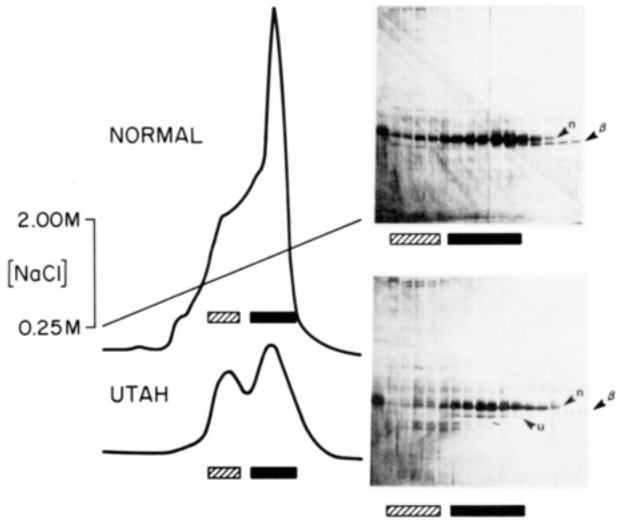

FIGURE 3: Heparin–Sepharose chromatography of normal and Utah plasma. Aliquots (100 mL) of normal or Utah plasma containing 9.1 and 9.2 g of total protein, respectively, were applied to a heparin–Sepharose column. The column was eluted with a linear 0.25–2.0 M NaCl gradient. A_{280} profiles are shown on the left, and silver-stained Laemmli gels of reduced and denatured aliquots from selected column fractions are presented on the right. The leftmost lane of each gel contains purified ATIII (Calbiochem). The crosshatched and black bars indicate how the column fractions and gels align. In the gel photographs, the band corresponding to the major ATIII species is marked n, that corresponding to the minor ATIII species, ATIII-β, is marked β, and that corresponding to ATIII-Utah is marked u. Like ATIII-Utah, ATIII-β has increased electrophoretic mobility relative to ATIIIn; this characteristic and the increased affinity of ATIII-β for heparin result from the absence of an N-linked oligosaccharide side chain on Asn-135 (Brennan et al., 1987).

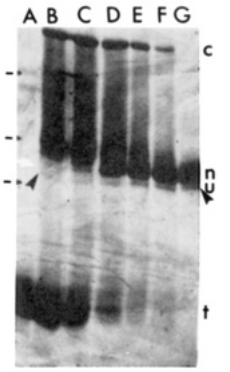

FIGURE 4: ATIII-Utah does not form protease–inhibitor complexes with thrombin. An antithrombin III fraction containing normal (n) and Utah (u) molecules was isolated from patient plasma by heparin–Sepharose chromatography. Aliquots (2 μg) of this heparin-activated ATIII preparation were incubated with varying amounts of human thrombin (t). Complex (c) formation was analyzed by SDS–polyacrylamide gel electrophoresis. Marks to the left of the gel indicate mobility of prestained 84 000-, 58 000-, and 48 500-dalton markers (Sigma SDS-7B). (A) 35 μg of thrombin. (B-G) 2 μg of patient ATIII plus thrombin as indicated (B) 17.5 μg, (C) 8.75 μg, (D) 4.375 μg, (E) 2.188 μg, (F) 1.094 μg, or (G) no thrombin. Black arrowheads point to the ATIII-Utah bands in lanes B and G. A white arrow points to the position in lane B where uncomplexed ATIII-n would migrate.

been observed for other single amino acid replacements (de Jong et al., 1978).

The interaction of ATIII-Utah and thrombin was examined in vitro. Patient antithrombin, chromatographically purified as above, was activated with heparin and then incubated with thrombin. Figure 4 shows the results of the complex formation experiment. The product of the normal ATIII gene (n) formed protease–inhibitor complexes (c) with thrombin (t), but that of the ATIII-Utah gene (u) did not.

Discussion

A mutation converting proline-407 to leucine has been identified 14 amino acids C-terminal to the reactive site arginine of antithrombin III Utah. This substitution is located in a region of the inhibitor that has been highly conserved during evolution of the serpin gene family; proline-407 is an invariant residue in 16 of the 17 serpin sequences shown in Figure 6. Conservation of proline-407 indicates that it is critical for maintaining the structural and functional integrity of serine protease inhibitors and is consistent with the identification of the Utah mutation at this position. The α1-antitrypsin S and Z mutations (Owen & Carrell, 1976; Jeppsson, 1976) are also examples of serpin mutations where substitutions at invariant residues led to the production of dysfunctional proteins.

Previous immunoblotting studies on Utah family plasmas (Bock et al., 1985) and the column chromatography experiment shown in Figure 3 indicate that the Utah mutation has quantitative and qualitative effects. The mutant gene product is found in Utah plasma at extremely reduced concentration (<5%) compared to the product of the normal ATIII allele (quantitative effect). Since the risk of hypercoagulability and thrombosis increases greatly when ATIII levels drop to less than 80% of normal, the phenotypic anticoagulant defect observed in Utah patients is most probably mediated through the *quantitative* effect of the ATIII-Utah mutation. Decreased plasma concentration of the ATIII-Utah gene product could be caused by increased susceptibility of an improperly folded molecule to proteolytic degradation. The tertiary structure of the homologous serpin α1-antitrypsin (Loebermann et al., 1984) can be used as a model for the tertiary structures of other serpin family members (Bock et al., 1986), including antithrombin III, and analysis of this model suggests that the Pro-407 to Leu substitution could lead to the production of an improperly folded inhibitor. The α1-antitrypsin structure was solved at 3A for the cleaved inhibitor and revealed a highly ordered globular molecule consisting of three sheets surrounded by nine helices. Proline-407 [equivalent to proline-369 of α1-antitrypsin (Figure 6)] is located at the junction of sheet strands 1C and 4B. In this position, the imino acid proline may play a crucial role in determining the geometry of the inhibitor core. Substitution of a leucine for proline-407 could cause an overall disruption of inhibitor structure and lead to more rapid turnover of ATIII-Utah.

Given that the plasma concentration of antithrombin III Utah is so severely reduced, this type II ATIII deficiency becomes in effect a type I deficiency, and it is physiologically irrelevant whether or not the small amount of circulating mutant inhibitor is functionally active or not. However, the question of whether or not ATIII-Utah retains the ability to act as an effective thrombin inhibitor is interesting from a biochemical point of view. It seemed unlikely that the antithrombin III Utah gene encoded an active protease inhibitor on the basis of the proximity of the mutation to the ATIII reactive site and the high degree of sequence conservation at proline 407 and in the region around it. Analytical gel investigation of this issue through complex formation experiments (Figure 4) confirmed that ATIII-Utah is a functionally defective protease inhibitor.

Since the anticoagulant effect of antithrombin III is increased over 1000-fold by heparin, the question of heparin binding by ATIII-Utah was investigated. ATIII-Utah elutes

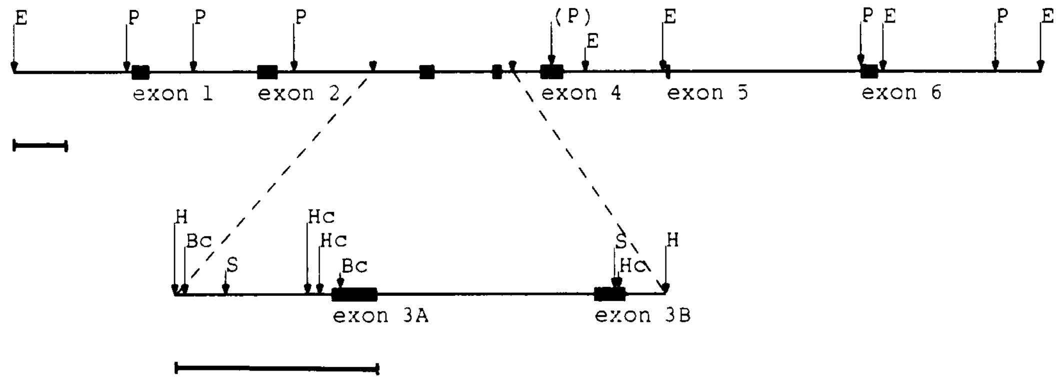

FIGURE 5: Revised structure of the normal human antithrombin III gene. Structure of the exon 3A–3B region (lower line) was determined as indicated in the text and Figure 1B. Structure of flanking regions (upper line) is from Prochownik et al. (1985). Restriction sites are Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; and S, *Sst*I. (P) indicates the polymorphic *Pst*I site described in Prochownik et al. (1983a). Scale bar under each map indicates 1 kb.

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            370                 380                 390     394
            <   sheet 4B    > <     sheet 5B        >
        * * * * * *   *                 * * * * * *   * *
ATIII:  N R P F L V F I R E V P L N T I I F M G R V A N P C V K
A1AT:   N K P F V F L M I E Q N T K S P L F M G K V V N P T Q K
C1inh:  Q Q P F L F M L W D Q Q H K F P V F M G R V Y D P R A
hcII:   D R P F L F L I Y E H R T S C L L F M G R V A N P S R S
A1ACT:  N R P F L M I I V P T D T Q N I F F M S K V T N P K Q A
A2AP:   N R P F L F F I F E D T T G L P L F V G S V R N P N P S A +51
ePAI:   D R P F L F V V R H N P T G T V L F M G Q V M E P
pPAI:   D H P F L F L I M H K I T K C I L F F G R F C S P
PCI:    N R P F L M F I V D N N - - - I L F L G K V N R P
GDN:    D R P F L F F I R H N P T G A V L F M G Q I N K P
AGTH:   N R P F L F A V Y D Q S A T A L H F L G R V A N P L S T A
TBG:    D R S F M L L I L E R S T R S I L F L G K V V N P T E A
ctpsn:  N R P F L F V I Y H T S A Q S I L F M A K V N N P K
ova:    D H P F L F C I K H I A T N A V L F F G R C V S P
bpZ:    N H P F L F L I R E D I A G V V V F V G H V T N P L I S A
CPV38:  D H P F I Y V I R H V D G K - I L F V G R Y C S P T T N
rORF1:  N K P F M F L I Y H K P T T T V L F M G K V I Y D T E G R +7
                                          T I T K G E
```

FIGURE 6: Alignment of the C-terminal amino acid sequences of some serpin gene family members. Highly conserved residues are marked with stars. The numbering shown is that of α1-antitrypsin, and the correspondence of structural elements from the cleaved α1-antitrypsin structure (Loebermann et al., 1984) with the amino acid sequences is marked above the alignment. The proline residue that is replaced by a leucine in antithrombin III Utah is in boldface type. Dashes indicate gaps in the sequences. Sequences were obtained from *The Atlas of Protein Sequence and Structure* with the following exceptions: A1ACT and contrapsin were from Hill et al. (1984), hcII was from Ragg (1986), bpZ was from Hejgaard et al. (1985), A2AP was from Holmes et al. (1987), ePAI was from Ny et al. (1986), pPAI was from Ye et al. (1987), PCI was from Suzuki et al. (1987), GDN was from Sommer et al. (1987), CPV38 was from Pickup et al. (1986), TBG was from Flink et al. (1986), and rORF1 was from Upton et al. (1986). The abbreviations used are A1AT, α1-antitrypsin; C1inh, C$\bar{1}$ inhibitor; hcII, heparin cofactor II; A1ACT, α1-antichymotrypsin; A2AP, α2-antiplasmin; ePAI, endothelial cell plasminogen activator inhibitor; pPAI, placental plasminogen activator inhibitor; PCI, protein C inhibitor; GDN, glial-derived protease nexin; AGTH, angiotensinogen; TBG, thyroxine binding globulin; ctpsn, contrapsin; ova, ovalbumin; bpZ, barley protein Z; CPV38, 38-kDa cowpox virus protein; and rORF1, rabbit plasmid open reading frame 1. Numbers at the end of some sequences indicate that they extend the indicated number of amino acid residues to their C termini.

from heparin–Sepharose at the same salt concentration as normal ATIII (Figure 3), indicating that the Pro-407 to Leu mutation has not affected its ability to bind to heparin. This observation in conjunction with data from the complex formation experiment discussed above indicates that the part(s) of the ATIII-Utah molecule that bind heparin and thrombin are distinct. The amino-terminal end of ATIII (Koide et al., 1982, 1984) and/or the G (Villaneuva, 1984) and/or A and D (Carrell et al., 1987) helices are hypothesized to be involved in heparin binding. Apparently, the structural perturbation caused by the proline-407 to leucine substitution does not adversely affect these areas or whatever region of the ATIII molecule is responsible for heparin binding.

The ATIII-Utah mutation destroyed a *Stu*I restriction site present in the DNA encoding Arg-406 and Pro-407 of the normal ATIII gene (Figure 2). This change permits rapid and accurate identification of the Utah mutation in *Stu*I digests of genomic DNA and will be useful for diagnosis of affected individuals in the Utah family.

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