Isolation and sequence characterization of a cDNA clone of human antithrombin III

(natural anticoagulant/plasma protease inhibitor/human liver cDNA library/molecular cloning/DNA sequence)

T. CHANDRA, ROBIN STACKHOUSE, VINCENT J. KIDD, AND SAVIO L. C. WOO*

Howard Hughes Medical Institute, Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Communicated by Earl W. Davie, December 29, 1982

ABSTRACT A human liver cDNA library was constructed by using poly(A)-containing RNA isolated from a human liver biopsy specimen. This library is comprised of 40,000 independent transformants with an average inserted DNA length of 1,200 base pairs. By using the previously cloned baboon antithrombin III cDNA as a specific hybridization probe, >30 human antithrombin III cDNA clones were identified from this library. The clone with the longest DNA insert was selected for sequence analysis. This antithrombin III cDNA clone contains 1,479 base pairs of inserted human DNA and was designated phATIII 113. It contains DNA sequences that code for a signal peptide and the entire mature antithrombin III protein which is comprised of 432 amino acid residues.

Antithrombin III is a plasma protease inhibitor synthesized in the liver. The glycoprotein has a molecular weight of 55,000 and its entire amino acid sequence has almost been completed (1). It is a natural anticoagulant in that it specifically inhibits a number of serine proteases that participate in the blood coagulation cascade, including thrombin, factors IXa, Xa, XIa, and XIIa (2–5). The mechanism of inhibition involves the stoichiometric formation of protease–antiprotease complexes and the rate of complex formation is greatly enhanced in the presence of heparin, which is a well-known anticoagulant used clinically in myocardial infarction and surgery (6, 7). Deficiency of antithrombin III is a hereditary disorder that is associated with recurrent thrombophlebitis, acute aortic thrombosis, and thromboembolism (8–10). Heterogeneity of the classical antithrombin III deficiency has been observed (11). Abnormal antithrombin III also has been isolated from deficient patients and partially characterized (12), suggesting that the deficiency could be the result of mutations in the antithrombin III gene itself. Therefore, the genetic deficiency can be analyzed in molecular detail if the human antithrombin III gene can be isolated and characterized. We recently have reported the purification of antithrombin III mRNA from a baboon liver by polysome immunoprecipitation and the cloning of its cDNA (13). In this paper, we report the construction of a human liver cDNA library and the identification and sequence of a full-length human antithrombin III cDNA clone.

MATERIALS AND METHODS

Construction of a Human Liver cDNA Library. A human liver biopsy specimen was kindly provided by Bill Williams (University of Texas Medical School in Houston). Total nucleic acid was extracted from the frozen tissue with phenol (14), and poly(A)-containing RNA was prepared by oligo(dT)-cellulose column chromatography (15). The RNA preparation

was used for cDNA synthesis under conditions that favored full-length cDNA production (16). The cDNA preparation was sedimented through an alkaline sucrose gradient (16) and only fractions containing cDNA species of 1,000 nucleotides or more were pooled. The single-stranded cDNA was subsequently made double-stranded with reverse transcriptase (17). After nuclease S1 treatment, tracts of poly(dC) were added to the 3' termini of the DNA molecules by using terminal transferase (18). The enzymatically synthesized cDNA was rehybridized with *Pst* I-linearized pBR322 DNA that had been tailed with poly(dG) and the DNA was used for transformation of *Escherichia coli* RR1 (18). Bacterial transformants were selected for resistance to tetracycline and ≈40,000 individual recombinants containing human liver cDNA sequences were obtained. Analysis of the lengths of the inserted DNA in 20 randomly selected colonies by minilysis (19) and electrophoresis has indicated that >95% of the transformants were indeed recombinants, and the average length of human DNA in these recombinants was ≈1,200 base pairs.

Identification of Human Antithrombin III cDNA Clones from a Human Liver cDNA Library. The human liver cDNA library was screened by colony hybridization (20) by using a nick-translated *Pst* I fragment from a baboon antithrombin III cDNA clone (13). Recombinants containing human antithrombin III DNA sequences were identified and the lengths of DNA inserts in these clones were analyzed by both agarose and polyacrylamide gel electrophoreses. The clone with the largest DNA insert, designated phATIII 113, was chosen for DNA sequence analysis by the method of Maxam and Gilbert (21).

RESULTS

By using human liver poly(A)-containing RNA and standard cloning procedures, a cDNA library comprised of 40,000 independent transformants with an average inserted DNA length of 1,200 base pairs was constructed. Screening of the library with a baboon antithrombin III cDNA clone (13) under stringent hybridization conditions led to the identification of >30 positive clones. Analysis of plasmid DNAs isolated from these human cDNA clones by a minilysis procedure and agarose gel electrophoresis showed that they contained common internal restriction fragments. The recombinant containing the largest DNA insert was selected for DNA sequence analysis. The clone, designated phATIII 113, contained about 1,500 base pairs of human DNA and was subjected to sequence analysis by the strategy shown in Fig. 1. Throughout most of the DNA molecules, both DNA strands were subjected to sequence analysis. When only one DNA strand was subjected to sequence analysis, at least two experiments were performed that involved the use of independent labeling sites. Finally, all la-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* To whom correspondence should be addressed.

FIG. 1. Sequence analysis strategy of the human antithrombin III cDNA clone. The restriction sites used for labeling are shown, but they do not represent all such sites present on the human DNA. The 5' ends of DNA fragments were labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ employing polynucleotide T4 kinase. The *Fnu4HI* site at the 3' end was labeled with the appropriate $[\alpha\text{-}^{32}\text{P}]\text{dNTP}$ by using the Klenow fragment of *E. coli* DNA polymerase.

beling and overlapping sites were subjected to sequence analysis to ensure the accuracy of the data.

The inserted human DNA sequence was 1,479 nucleotides in length and was flanked by poly(dG) and poly(dC) of 7 and 13 residues at the 5' and 3' termini, respectively (Fig. 2). Comparison of the DNA sequence with the known amino acid sequence of the protein (1) has shown that the codon for the amino-terminal histidine present in the mature plasma protein was located at nucleotides 97–99 in the cDNA clone. The rest of the amino acid sequence derived from the DNA sequence agrees perfectly with the published amino acid sequence of human antithrombin III (1). Finally, the amino acid sequence in a "gap" region of the protein (residues 214–221) has been determined to be Val-Leu-Val-Asn-Thr-Ile-Tyr-Phe from the DNA sequence.

The amino acid residue immediately preceding the first amino acid of the mature protein was not a methionine. Because antithrombin III is a secretory protein synthesized in the liver, the presence of a signal peptide at the NH_2 terminus of the protein could be expected for intracellular membrane transport (22). Indeed, the DNA sequence maintained an open reading frame at this region and the first methionine codon was located at the 5' terminus of the cDNA clone at position –32 (Fig. 2). If this methionine residue were the starting site, the signal peptide is 32 amino acids in length. However, another methionine residue further upstream cannot be ruled out as the starting site for the signal peptide.

In addition, DNA sequence analysis at the 3' terminus of this human antithrombin III clone has revealed a stretch of poly(dA) preceding the poly(dC) residues. This indicates that pATIII 113 contains human DNA sequences that code for all amino acid residues in antithrombin III, most if not all of the leader sequence, and all of the 3' untranslated region of the

mRNA. Like other eukaryotic mRNAs, it also contains the consensus hexanucleotide A-A-T-A-A sequence in close proximity to the 3' terminus which is necessary for polyadenylation of the RNA.

DISCUSSION

A human liver cDNA library has been screened by using a previously reported baboon antithrombin III cDNA clone (13) and >30 human antithrombin III cDNA clones were identified. The inserted human DNA in the largest recombinant plasmid was subjected to sequence analysis in its entirety and its corresponding amino acid sequence has been deduced. From the nucleotide sequence, it is apparent that human antithrombin III, being a plasma protease inhibitor synthesized in the liver, contains a signal peptide of 31 or more amino acids which is involved in intracellular transport through the endoplasmic reticulum (22).

Having cloned and characterized the human antithrombin III cDNA, it would be possible to analyze the familial antithrombin III deficiency by gene mapping, much in the same way as the cloned human α - and β -globin genes were utilized in the analysis of various hemoglobinopathies. This type of analysis has led to the development of gene mapping methodologies for prenatal diagnosis of various thalassemias and sickle cell anemia by genetic polymorphism linkage to the hereditary disorders (23, 24). Recently, our laboratory also has been able to use a cloned human phenylalanine hydroxylase cDNA probe to analyze classical phenylketonuria by restriction fragment-length polymorphism (unpublished data). Subsequently, methods for direct analysis of the point mutation in the sickle cell trait also have been developed, mainly by identification of restriction enzymes that could distinguish the

FIG. 2 (on following page). The complete nucleotide sequence of the human DNA insert in pATIII 113 and the amino acid sequence deduced from the DNA sequence. The amino acid residues in the mature protein are numbered 1 through 432, and those in the putative signal peptide are numbered –1 through –31, with the initiation codon for methionine as number –32. The numbering of the nucleotide sequence starts with the ATG initiation codon for the putative pre-antithrombin III protein.

-32 MET TYR SER ASN VAL ILE GLY THR VAL THR SER GLY LYS -20 ARG LYS VAL TYR LEU LEU SER
 (G)CA T G T A T T C C A A T G T G A T A G G A A C T G T A C C T C T G G A A A A G G A A G G T T T A T C T T T T G T C C
 10 20 30 40 50 60
 LEU LEU LEU ILE GLY PHE TRP ASP CYS VAL THR THR -1 1 HIS GLY SER PRO VAL ASP ILE CYS
 T T G C T G C T C A T T G G C T T C T G G G A C T G C G T G A C C T G T C A C G G A A G C C C T G T G A C A T C T G C
 70 80 90 100 110 120
 THR ALA LYS PRO ARG ASP ILE PRO MET ASN PRO MET CYS ILE TYR ARG SER PRO GLU LYS
 A C A G C C A A G C C G C G G G A C A T T C C C A T G A A T C C C A T G T G C A T T T A C C G C T C C C G G A G A A G
 130 140 150 160 170 180
 LYS ALA THR GLU ASP GLU GLY SER GLU GLN LYS ILE PRO GLU ALA THR ASN ARG ARG VAL
 A A G G C A A C T G A G G A T G A G G G C T C A G A A C A G A A G A T C C C G G A G G C C A C C A A C C G G C G T G T C
 190 200 210 220 230 240
 TRP GLU LEU SER LYS ALA ASN SER ARG PHE ALA THR THR PHE TYR GLN HIS LEU ALA ASP
 T G G G A A C T G T C C A A G G C C A A T T C C C G C T T T G C T A C C A C T T T C T A T C A G C A C C T G G C A G A T
 250 260 270 280 290 300
 SER LYS ASN ASP ASN ASP ASN ILE PHE LEU SER PRO LEU SER ILE SER THR ALA PHE
 T C C A A G A A T G A C A A T G A T A A C A T T T T C T G T C A C C C C T G A G T A T C T C C A C G G C T T T T G C T
 310 320 330 340 350 360
 MET THR LYS LEU GLY ALA CYS ASN ASP THR LEU GLN GLN LEU MET GLU VAL PHE LYS PHE
 A T G A C C A A G C T G G G T G C C T G T A A T G A C A C C C T C C A G C A A C T G A T G G A G G T A T T T A A G T T
 370 380 390 400 410 420
 ASP THR ILE SER GLU LYS THR SER ASP GLN ILE HIS PHE PHE PHE ALA LYS LEU ASN
 G A C A C C A T A T C T G A G A A A C A C A T C T G A T C A G A T C C A C T T C T T T T G C C A A A C T G A A C T G C
 430 440 450 460 470 480
 ARG LEU TYR ARG LYS ALA ASN LYS SER SER LYS LEU VAL SER ALA ASN ARG LEU PHE
 C G A C T C T A T C G A A A A G C C A A C A A A T C C T C C A A G T T A G T G T C A G C C A A T C G C C T T T T G G A
 490 500 510 520 530 540
 ASP LYS SER LEU THR PHE ASN GLU THR TYR GLN ASP ILE SER GLU LEU VAL TYR GLY ALA
 G A C A A A T C C C T T A C C T T C A A T G A G A C C T A C C A G G A C A T C A G T G A G T T G G T A T A T G G A G C C
 550 560 570 580 590 600
 LYS LEU GLN PRO LEU ASP PHE LYS GLU ASN ALA GLU GLN SER ARG ALA ALA ILE ASN
 A A G C T C C A G C C C C T G G A C T T C A A G G A A A A T G C A G A G C A A T C C A G A G C T G C C A T C A C A A A
 610 620 630 640 650 660
 TRP VAL SER ASN LYS THR GLU GLY ARG ILE THR ASP VAL ILE PRO SER GLU ALA ILE ASN
 T G G G T G T C C A A T A A G A C C G A A G G C G T A T C A C C G A T G T C A T T C C C T C G G A A G C C A T C A A T
 670 680 690 700 710 720
 GLU LEU THR VAL LEU VAL LEU VAL ASN THR ILE TYR PHE LYS GLY LEU TRP LYS SER LYS
 G A G C T C A C T G T T C T G G T G C T G G T T A A C A C C A T T T A C T T C A A G G G C C T G T G G A A G T C A A A G
 730 740 750 760 770 780
 PHE SER PRO GLU ASN THR ARG LYS GLU LEU PHE TYR LYS ALA ASP GLY GLU SER CYS SER
 T T C A G C C C T G A G A A C A C A A G G A A G G A A C T G T T T C T A C A A G G C T G A T G G A G A G T C G T G T C A
 790 800 810 820 830 840
 ALA SER MET MET TYR GLN GLU GLY LYS PHE ARG TYR ARG ARG VAL ALA GLU GLY THR GLN
 G C A T C T A T G A T G T A C C A A G A A G G C A A G T T C C G T T A T C G G C G C G T G G C T G A A G G C A C C C A G
 850 860 870 880 890 900
 VAL LEU GLU LEU PRO PHE LYS GLY ASP ASP ILE THR MET VAL LEU ILE LEU PRO LYS PRO
 G T G C T T G A G T T G C C C T T C A A A G G T G A T G A C A T C A C C A T G G T C T C A T C T T G C C C A A G C C T
 910 920 930 940 950 960
 GLU LYS SER LEU ALA LYS VAL GLU LYS GLU LEU THR PRO GLU VAL LEU GLN GLU TRP LEU
 G A G A A G A G C C T G G C C A A G G T A G A G A A G A A C T C A C C C C A G A G G T G C T G C A A G A G T G G C T G
 970 980 990 1000 1010 1020
 ASP GLU LEU GLU GLU MET MET LEU VAL VAL HIS MET PRO ARG PHE ARG ILE GLU ASP GLY
 G A T G A A T T G G A G G A G A T G A T G C T G G T G G T C C A C A T G C C C C G C T T C C G C A T T G A G G A C G G C
 1030 1040 1050 1060 1070 1080
 PHE SER LEU LYS GLU GLN LEU GLN ASP MET GLY LEU VAL ASP LEU PHE SER PRO GLU LYS
 T T C A G T T T G A A G G A G C A G C T G C A A G A C A T G G G C C T T G T C G A T C T G T T C A G C C C T G A A A A G
 1090 1100 1110 1120 1130 1140
 SER LYS LEU PRO GLY ILE VAL ALA GLU GLY ARG ASP ASP LEU TYR VAL SER ASP ALA PHE
 T C C A A A C T C C C A G G T A T T G T T G C A G A A G G C C G A G A T G A C C T C T A T G T C T C A G A T G C A T T C
 1150 1160 1170 1180 1190 1200
 HIS LYS ALA PHE LEU GLU VAL ASN GLU GLU GLY SER GLU ALA ALA ALA SER THR ALA VAL
 C A T A A G G C A T T T T G A G G T A A A T G A A G A A G G C A G T G A A G C A G C T G C A A G T A C C G C T G T T
 1210 1220 1230 1240 1250 1260
 VAL ILE ALA GLY ARG SER LEU ASN PRO ASN ARG VAL THR PHE LYS ALA ASN ARG PRO
 G T G A T T G C T G G C C G T T C G C T A A A C C C C A C A A G G G T G A C T T T C A A G G C C A A C A G G C C T T T C
 1270 1280 1290 1300 1310 1320
 LEU VAL PHE ILE ARG GLU VAL PRO LEU ASN THR ILE ILE PHE MET GLY ARG VAL ALA
 C T G G T T T T A T A A G A G A A B T T C C T C T G A A C A C T A T T A T C T T C A T G G G C A G A G T A G C C A A C
 1330 1340 1350 1360 1370 1380
 PRO CYS VAL LYS ***
 C C T T G T G T T A A G T A A A A T G T T C T T A T T C T T T G C A C C T C T T C T A T T T T G T T T G T G A A C
 1390 1400 1410 1420 1430 1440
 A G A A G T A A A A T A A A T A C A A A C T A C T T C C A T C T C A C A T T (U)_n (C)_n
 1450 1460 1470 1480

FIG. 2. (Legend appears at the bottom of the preceding page.)

mutated nucleotide in the β -globin gene (25, 26). In cases such as α_1 -antitrypsin deficiency, in which the point mutation does not create or destroy a restriction recognition sequence, specific oligonucleotides can be synthesized and used to distinguish the normal and mutated genes in chromosomal DNA (unpublished data). Thus, the cloning, sequence analysis, and comparison of the normal and deficient antithrombin III cDNAs should permit the development of such methodologies for prenatal diagnosis of antithrombin III deficiency. Furthermore, because phATIII 113 reported here apparently contains all of the peptide-coding sequences, it should be possible to use DNA engineering technology for production of this natural anticoagulant for therapeutic application in deficient individuals as well as patients with other coagulation complications.

Antithrombin III shares significant amino acid sequence homology with human α_1 -antitrypsin (1, 27, 28), which is the major plasma protease inhibitor that serves as the principal neutralizing factor for polymorphonuclear leukocyte elastase (29). Comparison of the nucleotide sequences between the human cDNA clones revealed a homology level of 46.5%. Further comparison of the two sequences by computer-assisted dot-matrix analysis (30) has not revealed any regions in the genes that are particularly homologous with each other and the sequence homology is spread rather evenly throughout the molecules. Surprisingly, the two plasma protease inhibitors also share significant sequence homology with chicken ovalbumin (28), which is the major egg-white protein and has no apparent protease inhibitor activity. The three proteins have been classified as members of a superfamily that had diverged over 500 million years ago (31). We recently have reported the cloning and characterization of the human chromosomal α_1 -antitrypsin gene (31). Comparison of its molecular structure with that of the chicken ovalbumin gene showed that the number, size, and positioning of the two sequence-related genes are completely different, suggesting that intronic sequences also could be inserted into preexisting exonic sequences if the two genes arose by divergent evolution (31). Because the extent of amino acid sequence homology between antithrombin III and α_1 -antitrypsin is greater than that between α_1 -antitrypsin and chicken ovalbumin, it would be interesting to examine the genomic organization of the antithrombin III gene and compare its molecular structure with those of the human α_1 -antitrypsin and chicken ovalbumin genes. These studies could lead to a better understanding of the evolutionary origin of this interesting gene family.

The authors are indebted to Dr. Bill Williams of the University of Texas Medical School at Houston for providing us with the human liver biopsy specimen for construction of the cDNA library. We also thank Dr. Charles Manner and Ms. Wanda Beattie for helpful discussion and Ms. Sharon Moore for excellent technical assistance. The work was partially supported by Grant HL-27509 from the National Institutes of Health, and S. L. C. W. is an Investigator of the Howard Hughes Medical Institute.

1. Peterson, T. E., Dudek-Wojciechowska, G., Scottrup-Jensen, L., & Magnusson, S. (1979) in *The Physiological Inhibitors of Blood*

- Coagulation and Fibrinolysis*, eds. Collen, D., Wiman, B. & Verstraete, M. (Elsevier/North-Holland, Amsterdam), pp. 43–54.
2. Heimburger, N., Haupt, H. & Schwick, H. G. (1971) in *Proceedings of the International Research Conference on Proteinase Inhibitors*, eds. Fritz, H. & Tschesche, H. (Degruyter, Berlin), pp. 1–22.
3. Osterud, B., Miller-Andersson, M., Abildgaard, U. & Prydz, H. (1976) *Thromb. Haemostasis* **35**, 295–305.
4. Kurachi, K., Fujikawa, K., Schmer, G. & Davie, E. W. (1976) *Biochemistry* **15**, 373–377.
5. Kurachi, K. & Davie, E. W. (1977) *Biochemistry* **16**, 5831–5839.
6. Damus, P. S., Hicks, M. & Rosenberg, R. D. (1973) *Nature (London)* **246**, 355–357.
7. Abildgaard, U. (1978) in *Proceedings of First Florence Conference on Hemostasis and Thrombosis*, eds. Neri Serneri, G. G. & Prentice, C. R. M. (Academic, London), pp. 169–176.
8. Egeberg, O. (1965) *Thromb. Diath. Haemorrh.* **13**, 516–530.
9. Shapiro, M. E., Rodvien, R., Bauer, K. A. & Salzman, E. W. (1981) *J. Am. Med. Assoc.* **245**, 1759–1763.
10. Arko, F. R., Jewell, C. T., Dainer, P. & MacLeod, W. A. J. (1979) *J. Am. Med. Assoc.* **242**, 2324–2325.
11. Sas, G., Peto, I., Banhegyi, D., Blasko, G. & Domjan, G. (1980) *Thromb. Haemostasis* **43**, 133–136.
12. Tran, T. H., Bounameaux, H., Bondeli, C., Honkanen, H., Marbet, G. A. & Duckert, F. (1980) *Thromb. Haemostasis* **44**, 87–91.
13. Stackhouse, R., Chandra, T., Robson, K. J. H. & Woo, S. L. C. (1982) *J. Biol. Chem.* **258**, 703–706.
14. Woo, S. L. C., Rosen, J. M., Liarakos, C. D., Choi, Y. C., Busch, H., Means, A. R. & O'Malley, B. W. (1973) *J. Biol. Chem.* **250**, 7027–7039.
15. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
16. Monahan, J. J., Harris, S. E., Woo, S. L. C. & O'Malley, B. W. (1976) *Biochemistry* **15**, 223–233.
17. Monahan, J. J., McReynolds, L. A. & O'Malley, B. W. (1976) *J. Biol. Chem.* **251**, 7355–7362.
18. Chandra, T., Kurachi, K., Davie, E. W. & Woo, S. L. C. (1981) *Biochem. Biophys. Res. Commun.* **103**, 751–758.
19. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
20. Grunstein, M. & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3961–3965.
21. Maxam, A. M. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
22. Jackson, R. C. & Blobel, G. (1980) *Ann. N.Y. Acad. Sci.* **343**, 391–403.
23. Kan, Y. W. & Dozy, A. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5631–5635.
24. Orkin, S. H., Kazazian, H. H., Antonarakis, S. E., Goff, S. C., Boehm, C. D., Sexton, J. P., Waber, P. G. & Giardina, P. J. V. (1982) *Nature (London)* **296**, 627–631.
25. Geever, R. F., Wilson, L. B., Wallaseth, F. S., Milner, P. F., Bittmer, M. & Wilson, J. T. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5081–5085.
26. Chang, J. C. & Kan, Y. W. (1981) *Lancet* **ii**, 1127–1129.
27. Kurachi, K., Chandra, T., Degen, S. J., White, T. T., Marchioro, T. L., Woo, S. L. C. & Davie, E. W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6826–6830.
28. Hunt, L. T. & Dayhoff, M. O. (1980) *Biochem. Biophys. Res. Commun.* **95**, 864–871.
29. Beatty, K., Bieth, J. & Travis, J. (1980) *J. Biol. Chem.* **255**, 3931–3934.
30. Novotny, T. (1982) *Nucleic Acids Res.* **10**, 127–131.
31. Leicht, M., Long, G. L., Chandra, T., Kurachi, K., Kidd, V. J., Mace, M., Jr., Davie, E. W. & Woo, S. L. C. (1982) *Nature (London)* **297**, 655–659.