Antithrombin III mRNA was enriched from a baboon liver by specific polysome immunoprecipitation. The partially purified antithrombin III mRNA preparation was used for cDNA synthesis and cloning. Candidate antithrombin III cDNA clones were identified by differential hybridization using as probes [32P]cDNAs synthesized from the polysome-enriched and -depleted RNA fractions, respectively. The candidate clones were further analyzed by hybrid-selected translation. The authenticity of a cDNA clone positive to both tests was unambiguously confirmed by matching its nucleotide sequence with the known amino acid sequence of human antithrombin III. The baboon antithrombin III cDNA clone hybridized well with human antithrombin III mRNA and can be used as a probe to isolate the corresponding human gene.

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 is known (1). It is a natural anti-coagulant in that it specifically inhibits a number of serine proteases in the coagulation cascade, including thrombin, factors VIIa, IXa, Xa, and XIIa (2-5). Its activity is enhanced tremendously after binding to heparin which is a well known anti-coagulant 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 has also 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. The genetic deficiency, therefore, can be analyzed in molecular detail if the antithrombin III gene can be isolated and characterized. As a first step toward this goal, we report the purification of antithrombin III mRNA and the cloning of its cDNA.

MATERIALS AND METHODS

Purification of Human Antithrombin III and Its Antibody—Antithrombin III was partially purified from human plasma by column chromatography using Bio-Rad Affi-Gel blue (13). Column fractions were analyzed for the presence of antithrombin III by immunodiffusion (14) using a rabbit anti-serum against human antithrombin III purchased from Sigma. The partially purified antithrombin III preparation was covalently linked to cyanogen bromide-activated Sepharose (Pharmacia). Specific immunoglobulin molecules against human antithrombin III were purified from the crude rabbit antiserum and rendered ribonuclease free by antigen affinity column chromatography (15).

Purification of Antithrombin III mRNA by Polysome Immunoprecipitation—Polysomes were extracted from a baboon liver according to a previously reported procedure (16). Polysomes engaged in antithrombin III synthesis were enriched from total liver polysomes by specific immunoprecipitation using affinity purified antibody against antithrombin III and Staphylococcus aureus cells (15). RNA was released from the bound polysome by treatment with sodium dodecyl sulfate/EDTA. Polyadenylate-containing RNA was isolated by oligo(dT)-cellulose column chromatography (17). Enrichment of specific antithrombin III mRNA by this procedure was assessed by cell-free translation in an mRNA-dependent rabbit reticulocyte lysate system (18) in the presence of [35S]methionine, followed by immunoprecipitation from the total translation products (19). Total and immunoprecipitated translation products were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (20) and radioactive protein bands were visualized by fluorography (21).

Synthesis and Cloning of Baboon Antithrombin III cDNA—Previously reported procedures were employed for the synthesis of cDNA from enriched antithrombin III mRNA, and its insertion into the PstI site of pBR322 (16). Identification of antithrombin III cDNA clones by differential hybridization and hybrid-selected translation were as described (22). The positive clone was further analyzed by DNA sequencing (23). These experiments were performed according to the guidelines for recombinant DNA research from the National Institutes of Health.

RESULTS

Enrichment of Baboon Antithrombin III mRNA by Polysome Immunoprecipitation—Antithrombin III mRNA constitutes about 0.1% of total liver mRNA. Cell-free translation of baboon liver polyadenylate-containing RNA showed an array of proteins of different molecular weights on a denaturing polyacrylamide gel (Fig. 1A, lane 4), and only minute quantities of antithrombin III were detectable by immunoprecipitation of the translation products with the specific immunoglobulins against antithrombin III (Fig. 1B, lane 4). A preparation of baboon liver polysomes was used for enrichment of antithrombin III mRNA by specific polysome immunoprecipitation. Cell-free translation of this mRNA preparation has shown that there was a marked decrease in total translational activity (Fig. 1A, lane 2), but immunoprecipitation of these products yielded a distinct antithrombin III band (50,000 daltons) plus 2 apparently truncated antithrombin III protein products (Fig. 1B, lane 4). Polysomal RNA depleted of antithrombin III mRNA produced a pattern similar to that of total polysomal RNA from baboon liver (Fig. 1A, lane 3), and no antithrombin III was immunoprecipitable from these translation products (Fig. 1B, lane 3). The enrichment for antithrombin III mRNA from total liver polysomes achieved by specific immunoprecipitation is estimated to be at least 50-fold.

Construction and Identification of a Baboon Antithrombin III Clone—Single-stranded cDNA was synthesized from 3 μg of polysome-enriched antithrombin III mRNA and centrifuged through an alkaline-sucrose gradient. Fractions containing DNA longer than 1000 nucleotides was made double-
Cloning of Antithrombin III cDNA

stranded and inserted into the PstI site of pBR322 by the dG/dC homopolymer addition method. Candidate antithrombin III cDNA clones were identified among recombinants by differential hybridization. Recombinant plasmid DNAs were prepared by the mini-lysate procedure (24), digested with BamHI and electrophoresed in an agarose gel. The gel was bi-directionally transferred to 2 nitrocellulose filters (25). The duplicate filters were hybridized separately with [32P]cDNA probes synthesized from polysome-enriched (Fig. 2A) and -depleted (Fig. 2B) mRNA preparations. DNAs in lanes 1 and 6 hybridized strongly with both probes, while DNA in lanes 2 and 5 hybridized moderately with both probes. DNAs in lanes 3 and 4, however, hybridized strongly with the antithrombin III-enriched probe but only moderately with the antithrombin III-depleted probe. These are, therefore, candidate clones to contain baboon antithrombin III DNA sequences.

Candidate antithrombin III cDNA clones were further analyzed by hybrid-selected translation. Immunoprecipitation of translation products from total baboon liver mRNA again showed very little radioactive antithrombin III (Fig. 3, lane 6), while the polysome-enriched polysomal mRNA again yielded a 50,000-dalton band plus truncated translation product (Fig. 3, lane 3). As a positive control for hybrid selection, a previously cloned baboon α1-antitrypsin cDNA (26) was also immobilized on to DBM-cellulose and used for hybrid selection. Immunoprecipitation of the translation products with anti-α1-antitrypsin immunoglobulins yielded a strong α1-antitrypsin band as expected (Fig. 3, lane 2). While protein synthesized from RNA selected by the cloning vector pBR322 showed no immunoprecipitable product with immunoglobulins against antithrombin III (Fig. 3, lane 1), RNA selected by one of the candidate recombinant clones (pbAT III) was apparently enriched for antithrombin III mRNA as indicated by the presence of an immunoprecipitable 50,000-dalton antithrombin III band (Fig. 3, lane 4).

Confirmation of the Baboon Antithrombin III cDNA Clone...
Cloning of Antithrombin III cDNA

with the published human antithrombin III sequence (Fig. 4). The two sequences were identical to each other for 37 out of 40 residues compared, and this cDNA clone apparently contained antithrombin III DNA sequences that coded for amino acid residues 71 to the COOH terminus of the protein. Discrepancies at positions 79 (Lys versus Asn) and 100 (Lys versus Gla) could represent genuine amino acid substitutions in the antithrombin III genes of the two species (Fig. 4).

**DISCUSSION**

Antithrombin III mRNA has been enriched from total polysomes of a baboon liver by specific immunoprecipitation and used for cloning of its cDNA. A genuine baboon AT III cDNA clone has been identified by differential hybridization, hybrid-selected translation, and DNA sequencing. Since an mRNA (data not shown), cDNAs should permit the development of such methodologies for prenatal diagnosis (5, 18).

Since the baboon antithrombin III cDNA clone hybridized well with human antithrombin III mRNA (data not shown), it could be used as a hybridization probe to isolate the corresponding human gene and analyze the familial antithrombin III deficiency by gene mapping. This type of analysis has led to the development of methodologies for prenatal diagnosis of various thalassemias and sickle cell anemia by genetic polymorphism linkage to the hereditary disorders (27, 28). More recently, methods for direct analysis of the point mutation in the sickle cell trait have also been developed, mainly by identification of restriction enzymes that could distinguish the mutated nucleotide in the β-globin gene (29, 30). The cloning, sequencing, and comparison of the normal and deficient antithrombin III cDNAs should permit the development of such methodologies for prenatal diagnosis of antithrombin III deficiency.

Antithrombin III shares significant amino acid sequence homology with human α1-antitrypsin (2, 26), which is another plasma protease inhibitor, and with chicken ovalbumin (31), which is the major egg white protein that apparently has no protease inhibitor activity. The 3 proteins had been classified as members of a super family that had diverged 500 million years ago (31). We have recently reported the cloning and characterization of the human chromosomal α1-antitrypsin gene (32). 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 could also be inserted into pre-existing exonic sequences if the two genes arose by divergent evolution (32). Since the extent of sequence homology between antithrombin III and α1-antitrypsin is greater than that between α1-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 α1-antitrypsin and chicken ovalbumin genes. These studies could lead to a better understanding on the evolutionary origin of this interesting gene family.

Acknowledgments—We would like to thank Dr. Charles Manner and Wanda Beatie for helpful discussion and Sharon Moore for excellent technical assistance.

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