

Evaluation of "at risk" alpha 1-antitrypsin genotype SZ with synthetic oligonucleotide gene probes.

T Nukiwa, ... , J P LeCocq, R G Crystal

J Clin Invest. 1986;77(2):528-537. <https://doi.org/10.1172/JCI112333>.

Research Article

Alpha 1-antitrypsin (alpha 1AT), a 52,000-mol-wt serum glycoprotein produced by hepatocytes and mononuclear phagocytes, functions as the major inhibitor of neutrophil elastase. The alpha 1AT haplotype S is associated with childhood liver disease and/or adult emphysema when inherited with the Z haplotype to give the phenotype SZ. To accurately identify the SZ phenotype at the level of genomic DNA, four ³²P-labeled 19-mer synthetic oligonucleotide probes were prepared; two to identify the M and S difference in exon III, and two to identify the M and Z difference in exon V. These probes were hybridized with various cloned DNAs and genomic DNAs cut with the restriction endonucleases BglII and EcoRI; the genomic DNAs represented all six possible phenotype combinations of the M, S, and Z haplotypes (MM, MS, MZ, SS, ZZ, and SZ). Using the four probes to evaluate 42 samples of genomic DNA, the "at risk" SZ and ZZ phenotypes were correctly identified in all cases, as were the "not at risk" phenotypes SS, MS, MM, and MZ, demonstrating that both exon III and exon V directed probes are necessary to properly identify all of the major "at risk" alpha 1AT genes. However, when used to evaluate a very rare family carrying a null allele, these four oligonucleotide probes misidentified the "at risk" null-null and S null phenotypes as "not [...]"

Find the latest version:

<https://jci.me/112333/pdf>



Evaluation of "At Risk" Alpha 1-Antitrypsin Genotype SZ with Synthetic Oligonucleotide Gene Probes

Toshihiro Nukiwa, Mark Brantly, Robert Garver, Lester Paul, Michael Courtney, Jean-Pierre LeCocq, and Ronald G. Crystal
Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892,
and Transgene SA, Strasbourg, France

Abstract

Alpha 1-antitrypsin (α 1AT), a 52,000-mol-wt serum glycoprotein produced by hepatocytes and mononuclear phagocytes, functions as the major inhibitor of neutrophil elastase. The α 1AT haplotype S is associated with childhood liver disease and/or adult emphysema when inherited with the Z haplotype to give the phenotype SZ. To accurately identify the SZ phenotype at the level of genomic DNA, four 32 P-labeled 19-mer synthetic oligonucleotide probes were prepared; two to identify the M and S difference in exon III, and two to identify the M and Z difference in exon V. These probes were hybridized with various cloned DNAs and genomic DNAs cut with the restriction endonucleases BglII and EcoRI; the genomic DNAs represented all six possible phenotype combinations of the M, S, and Z haplotypes (MM, MS, MZ, SS, ZZ, and SZ). Using the four probes to evaluate 42 samples of genomic DNA, the "at risk" SZ and ZZ phenotypes were correctly identified in all cases, as were the "not at risk" phenotypes SS, MS, MM, and MZ, demonstrating that both exon III and exon V directed probes are necessary to properly identify all of the major "at risk" α 1AT genes. However, when used to evaluate a very rare family carrying a null allele, these four oligonucleotide probes misidentified the "at risk" null-null and S null phenotypes as "not at risk" MM and SM combinations. These observations indicate that oligonucleotide gene probes yielded reliable and accurate assessment of "at risk" α 1AT genotypes in almost all situations, but in the context of prenatal diagnosis and genetic counseling this approach must be used with caution and in combination with family studies so as not to misidentify rare genotypes that may be associated with a risk for disease.

Introduction

Alpha 1-antitrypsin (α 1AT)¹, a 52,000-mol-wt serum glycoprotein produced by hepatocytes and mononuclear phagocytes, functions to inhibit neutrophil elastase, a proteolytic enzyme able to destroy all protein components of connective tissue (1–7). The α 1AT gene is highly pleomorphic; more than 30 different haplotypes have been described (3, 6). The α 1AT phenotype, referred to as the protease inhibitor (Pi) type, represents the co-dominant expression of the two parental α 1AT haplotypes (3).

Address reprint requests to Dr. Crystal, Building 10, Room 6D-03, National Institutes of Health, Bethesda, MD 20892.

Received for publication 7 August 1985.

1. Abbreviations used in this paper: α 1AT, alpha 1-antitrypsin; kb, kilobase; Pi, protease inhibitor; SSC, $1 \times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0.

The Journal of Clinical Investigation, Inc.
Volume 77, February 1986, 528–537

The most common α 1AT haplotypes in the USA are those of the M-family (M_1 , M_2 , M_3 ; combined haplotype frequency >0.90), the S type (frequency, 0.02–0.04), and the Z type (0.01–0.02) (8–11). The clinical interest in these α 1AT haplotypes is based on the knowledge that inheritance of the phenotypes PiZZ and PiSZ is associated with an increased risk for the development of emphysema (in adults) and/or liver disease (in children) (12–17). Studies at the gene and protein levels have shown that the S and Z genes each code for proteins that differ from the M protein by a single amino acid; in the S protein val²⁶⁴ replaces glu²⁶⁴, while in the Z protein lys³⁴² replaces glu³⁴² (18–20).

The classic approach to detecting the S and Z genes is at the protein level through a combination of serum isoelectric focusing, serum α 1AT levels, and family studies (21). However, such studies are often limited by the difficulty in establishing inheritance patterns (e.g., when serum cannot be obtained because family members are deceased or unavailable) or in the context where it is difficult or risky to obtain serum such as for prenatal diagnosis. To circumvent this problem, Kidd et al. (22) developed an approach utilizing synthetic oligonucleotide probes 19 bases in length that, under appropriate hybridization conditions, detected single base differences in the α 1AT gene in genomic DNA. Utilizing this approach, they showed that by using two oligonucleotide probes, one complementary to the M gene sequence in exon V and the other complementary to the Z gene sequence in exon V, the homozygous M (PiMM), homozygous Z (PiZZ), and heterozygous MZ (PiMZ) states could be distinguished (22, 23).

In this context, and in the context of the frequency of the S gene and its potential importance in defining the risk for disease, the present study extends oligonucleotide analysis of the α 1AT gene to include the S gene such that all possible "at risk" α 1AT phenotypes involving the S, Z, and M genes can be accurately identified. By evaluating 42 individuals with a variety of known α 1AT phenotypes, the data show that, with four oligonucleotide probes, two complementary to the M and Z difference in exon V, respectively, and two complementary to the M and S difference in exon III, respectively, accurate diagnosis of all combinations of the S, Z, and M genes are possible at the level of genomic DNA. Most importantly, using four oligonucleotide probes, it is possible to distinguish the "at risk" SZ heterozygote from the "not at risk" MS and MZ heterozygote states, a distinction that has major implications for prenatal diagnosis.

Methods

Study population. The study population consisted of 42 unrelated individuals. Grouped by their α 1AT serum phenotypes (see below), the population included: MM ($n = 11$; $M_1M_1 = 5$, $M_1M_2 = 4$, $M_1M_3 = 2$); SS ($n = 1$); ZZ ($n = 12$); MS ($n = 6$; $M_1S = 4$, $M_2S = 1$, $M_3S = 1$); MZ ($n = 8$; $M_1Z = 5$, $M_2Z = 2$, $M_3Z = 1$); and SZ ($n = 4$).

In addition, one family was evaluated in which both the S gene and null gene were carried. The index case that led to the identification of

this family was an individual with the null-null phenotype with no detectable α 1AT in the serum. This individual, a 35-yr-old with moderately severe emphysema in the absence of a history of smoking cigarettes, has been reported elsewhere (24). The phenotypes of the family members evaluated included: M_1M_2 ($n = 1$); $Mnull$ ($n = 4$; $M_1null = 1$, $M_2null = 3$); M_1S ($n = 1$); and $Snull$ ($n = 2$).

Determination of α 1AT phenotype. α 1AT phenotypes (referred to as the "Pi" type) were determined by a combination of isoelectrofocusing, serum α 1AT levels, and family studies (21). The isoelectrofocusing of the serum in polyacrylamide at pH 4 to 5 (Serva Fine Biochemicals, Inc., Garden City Park, NY; and Pharmacia Fine Chemicals, Piscataway, NJ) was carried out to achieve maximum separation of α 1AT M subtypes using a modification of the method of Constans et al. (25, 26). α 1AT serum levels were performed by radial immunodiffusion (Calbiochem-Behring Corp., La Jolla, CA) according to manufacturer's specification and standards. To avoid confusion with the clinical literature, all serum values reported here will be based on the same commercially available standards, although we, and others (27), recognize that the available α 1AT standards used by clinical laboratories overestimate α 1AT serum levels by $\sim 35\%$. Assignment of α 1AT protein phenotype was based on the international nomenclature classification (21).

Preparation of human genomic DNA. Human genomic DNA was prepared from peripheral white blood cells using a modification of the method of Jeffreys et al. (28). Briefly, 40 ml of blood was collected into heparinized tubes, followed by mixing with plasmagel (Cellular Products, Inc., Buffalo, NY) in a 1:1 ratio and allowed to separate into two phases at room temperature for 1 h. The upper layer containing white blood cells was centrifuged, and resulting cell pellet was washed and stored at -70°C . The frozen cell suspension was thawed, lysed, and subjected to phenol-chloroform extraction and ethanol precipitation. The DNA precipitate was dissolved in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, followed by RNAase A and proteinase K digestion and again subjected to phenol-chloroform extraction and ethanol precipitation. The final DNA preparation was dissolved in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. The purity and concentration of the final DNA preparation were determined by spectrophotometry.

Cloning of human α 1AT genes. Three human α 1AT genomic clones were used in this study, including: (a) λ ATMa14.4, a 14.4-kilobase (Kb) fragment of a human genomic DNA Charon 4A lambda phage library prepared by Lawn et al. (29); (b) λ ATMa17.5, a 17.5-kb fragment of the Lawn et al. library; and (c) λ ATM₂, a 10-kb fragment of a human DNA λ WES lambda phage library constructed from an individual known to have α 1AT PiM₂M₂. All clones were identified using a full-length α 1AT complementary DNA (cDNA) clone (plasmid pTG603) originally isolated by Courtney et al. (30) from a human liver cDNA library by screening with a synthetic oligonucleotide probe. The PstI insert was isolated, labeled with ^{32}P by nick translation (Amersham Corp., Arlington Heights, IL) and used to screen the genomic DNA libraries.

From the studies of Long et al. (20) it is known that the human genomic DNA library constructed by Lawn et al. (29) contains the S α 1AT gene. Thus, to provide a control for the use of oligonucleotide probes to evaluate the S α 1AT gene, the Lawn et al. (29) library was screened for fragments containing the α 1AT gene. This was accomplished by evaluating 10^6 plaque-forming unit of the library, using the α 1AT cDNA, pTG603, as a probe. Seven clones were isolated. Six contained a 14.4-kb insert of exons I to V inserted 3' to 5' in the phage. One phage, λ ATMa14.4, was selected for further study. A seventh clone was isolated that contained 17.5-kb insert that included (5' to 3') exons II to V and the 3' flanking region of the α 1AT gene; this clone was designated λ ATMa17.5. The phage containing α 1AT genes were grown in the large scale cultures, precipitated by PEG 8000, and purified by a CsCl stepwise density centrifugation and equilibrium banding (31). Purified phage particles were disrupted and digested with proteinase K at 65°C , 1 h, the DNA was extracted with phenol-chloroform, and then dialyzed against 10 mM Tris-HCl, pH 7.6, 1 mM EDTA and the amount of DNA determined by spectrophotometry.

To provide a genomic DNA standard of a known normal M gene, a phage library was constructed from genomic DNA of an individual

with the α 1AT serum phenotype Pi M₂M₂ (i.e., homozygous for the M₂ α 1AT gene; isoelectrofocusing Pi type M₂, serum α 1AT level 210 mg/dl). To accomplish this, high molecular weight DNA was extracted from peripheral blood white blood cells as described above. DNA (500 μg) was completely digested with the restriction endonuclease Eco RI (1 U/ μg DNA) for 4 h and digested DNA was applied on a preparative 0.8% agarose gel, 1.0 cm diameter $\times$ 3.0 cm length (PrepGel; Bethesda Research Laboratories, Gaithersburg, MD) and electrophoresed 30 V for 48 h. DNA fragments were fractionated by size (2 ml/tube, flow rate 2 ml/h). Fractions containing the 10.0-kb EcoRI fragment (exon II to V) of the α 1AT gene (see map, Fig. 1) were detected by hybridization with ^{32}P -labeled α 1AT cDNA as a probe. The DNA fraction (0.5 μg) containing the 10.0 kb EcoRI fragment was packaged in vitro into λ gtWES phage using a kit supplied by Amersham Corp. Starting from 10^5 plaque-forming unit lambda phages, one clone was isolated using ^{32}P -labeled α 1AT cDNA as a probe. This clone, designated λ ATM₂, was demonstrated by restriction mapping with ^{32}P -labeled cDNA to contain the 10.0-kb EcoRI insert of the α 1AT gene containing exons II to V.

Preparation of ^{32}P -labeled oligonucleotide probes. 19-mer oligonucleotides complementary to the region of exon III centered about the normal and mutated sequence of the S gene and the region of exon V centered about the normal and mutated sequence of the Z gene were synthesized (Pharmacia, Inc., Piscataway, NJ) with the mutated base in the center of the 19-mer. These 19-mers were used as templates for the labeling reaction (below). To prepare labeled probe to these regions, two oligonucleotides, the sequence of which is complementary to the 3' end of the respective exon III and exon V probes, were used as primers (see Fig. 1). Labeling of 19-mer oligonucleotides was performed by primer extension using *Escherichia coli* DNA polymerase I (Klenow fragment) by the method of Studencki et al. (32). Briefly, the 5' end of the primer oligonucleotides was first phosphorylated by T4 kinase according to the instruction of the supplier (5' DNA terminus labeling system, Bethesda Research Laboratories). [α - ^{32}P]dATP (6,000 Ci/mmol, 250 μCi , 36 pmol), [α - ^{32}P]dCTP (3,000 Ci/mmol, 125 μCi , 42 pmol), [α - ^{32}P]dGTP (3,000 Ci/mmol, 125 μCi , 42 pmol), and [α - ^{32}P]TTP (3,000 Ci/mmol, 125 μCi , 42 pmol) were dried, and a mixture of primer extension reaction reagents (50 mM NaCl, 25 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.72 μM template [19-mer], 2.7 μM primer [phosphorylated 8-mer], and 2.3 units *E. coli* DNA polymerase I [Klenow fragment]) was added to a final volume of 5 μl . The reaction was performed at 4°C for 3 h and terminated with the addition of 5 μl of a denaturing dye mixture (0.05% xylene cyanol, 0.05% bromophenol blue, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 8 M urea). The reaction mixture was then applied on 20% polyacrylamide 8 M urea gel (34 cm $\times$ 40 cm $\times$ 0.4 mm) and electrophoresed at 1,200 V, for 12 h. After autoradiography of the gel for 30 s, we identified and excised the location of the labeled 19-mer oligonucleotides (5 mm width). The excised gel was placed in 1 ml of 10 mM Tris-HCl, pH 7.6, and 1 mM EDTA for 2 h, centrifuged, and the supernatant containing the ^{32}P -labeled oligonucleotide probes was stored at -20°C . The separation of the labeled from the unlabeled probe was possible because the 8-mer primers were phosphorylated at the 5' terminus and the elongated 19-mer migrated faster than unphosphorylated 19-mer templates on the 20% polyacrylamide-8 M urea gel. Thus, the purified probe had a very high specific activity (10^8 dpm/pmol; 2×10^{10} dpm/ μg).

Strategy for evaluation of S mutation using oligonucleotide gene probes. The strategy used for the evaluation of the S mutation of the α 1AT gene was a modification of the approach used by Kidd et al. (22) to evaluate the Z mutation. Briefly, the oligonucleotide probes were designed to be complementary to 19 bases of the region of interest, with the base coding for the single base mutation at the center of the probe. Instead of using the restriction endonuclease HindIII and XbaI employed by Kidd et al. (22), we used double digestion with EcoRI and BglII; together these endonucleases cut the α 1AT gene such that the five exons are each contained in a separate fragment (Fig. 1). Thus, when electrophoresed on an agarose gel by size, the BglII-EcoRI digest results in the five exons being displayed individually in the following DNA fragments: 4.3 kb (exon II), 2.0 kb (exon I), 1.5 kb (exon IV), 1.3 kb (exon V), and 0.9 kb (exon III). With this approach, the S mutation in exon III can be identified

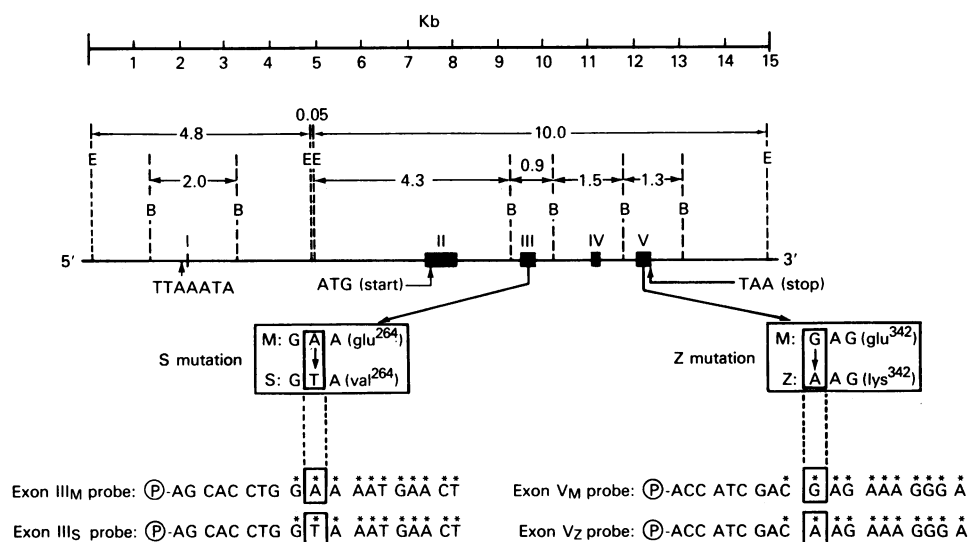


Figure 1. Strategy used to discriminate the S and Z mutations in the $\alpha 1$ AT antitrypsin gene using synthetic oligonucleotide gene probes. Shown is the structure of the $\alpha 1$ AT gene including five exons (solid boxes labeled I–V), the putative promoter site (TTAAATA) 5' to exon I, the start codon (ATG) in exon II, and the stop codon (TAA) in exon V. Using a combination of the endonucleases EcoRI (E) and BglII (B), the five exons composing the $\alpha 1$ AT gene are conveniently cut out in five DNA fragments, each of which contained one exon including: 2.0-kb fragment that includes exon I; 4.3-kb fragment with exon II; 0.9-kb fragment with exon III; 1.5-kb fragment with exon IV; and 1.3-kb fragment with exon V. Thus, on an autoradiogram of a Southern gel of the EcoRI-

BglII cut $\alpha 1$ AT gene, the DNA fragments of 4.3, 2.0, 1.5, 1.3, and 0.9 Kb, respectively, correspond to exons II, I, IV, V, and III. The S point mutation in exon III is caused by a base change of adenosine to thymidine causing amino acid glu²⁶⁴ to be altered to val²⁶⁴. The 19-mer oligonucleotide gene probes used to detect this change are indicated as "exon III_M probe" and "exon III_S probe" for the sequence in exon III of the M gene and S gene, respectively. The * indicates the bases in the probes that are labeled with ³²P (see Methods). Note that the oligonucleotide probes are designed with the single base difference in the center. The primers used to prepare the probe are phosphorylated ("P") at the 5' end. The Z point mutation in exon V is caused by a base change of guanosine to adenosine causing amino acid glu³⁴² to be altered to lys³⁴². Shown are the sequences of the oligonucleotide gene probes used to detect these sequences "exon V_M probe" and "exon V_Z probe," respectively.

by the presence or absence (depending on the probe) of a signal on the autoradiogram at a position that is specific for exon III.

All evaluations were carried out using four oligonucleotide probes. Two probes (referred to as "exon III_M probe" and "exon III_S probe") were used to evaluate the 19 base region of exon III centered at the A (M gene) to T (S gene) alteration that defines the S mutation, giving rise to the change of glu²⁶⁴ to val²⁶⁴ in the $\alpha 1$ AT protein. Two probes (referred to as "exon V_M probe" and "exon V_Z probe") were used to evaluate the 19 base region of exon V centered at the G (M gene) to A (Z gene) alteration that defines the Z mutation, giving rise to the change of glu³⁴² to lys³⁴² in the $\alpha 1$ AT protein.

Hybridization and autoradiography using oligonucleotide probes. Human genomic DNA (20–50 μ g) were digested with restriction endonuclease BglII (1 U/ μ g of DNA) for 8 h, 37°C, and then endonuclease EcoRI (1 U/ μ g DNA) for 3 h, 37°C, under the condition indicated by the supplier. Digested genomic DNA was extracted with phenol-chloroform, precipitated with ethanol, and redissolved in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA to make the concentration of DNA $\sim$ 1 mg/ml.

5 μ g per lane of the digested and concentrated DNA were electrophoresed on 1% agarose gel at 25 V for 16 h. The DNA in the gel was denatured with 0.5 M NaOH-1 M NaCl for 45 min, neutralized with 0.5 M Tris-HCl, pH 5.5, 3 M NaCl for 45 min, and subjected to blotting onto a nitrocellulose membrane by the method of Southern (33). The nitrocellulose sheet was baked at 80°C, 2 h, and stored at 4°C until use.

Before hybridization, blotted and baked nitrocellulose sheets were exposed to 100 ml of 6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution = 0.2 g Ficoll, 0.2 g polyvinylpyrrolidone, and 0.2 g bovine serum albumin [Pentax Fraction V] in 1 liter H₂O), 0.1% sodium dodecyl sulfate, 0.05% sodium pyrophosphate at 65°C for 4 h. Hybridization with the ³²P-labeled 19-mer oligonucleotide (10⁶ dpm/ml) was then carried out with 10 ml of 6 $\times$ SSC, 10 $\times$ Denhardt's solution, and 0.05% sodium pyrophosphate at 53°C for 18 h. This was followed by washing (6 $\times$ SSC, 0.05% sodium pyrophosphate) as follows: two times shaking for 30 min at 23°C, 30 min with shaking at 37°C, 3 min at 55°C, and 30 min at 23°C. The hybridized nitrocellulose sheet was sealed and set into X-omatic cassette and autoradiographed with XAR-2 film (Eastman Kodak Co., Rochester,

NY) at -70°C for 2–4 h for cloned DNA and for 2–7 d with genomic DNA.

Results

Identification of the genotype of cloned $\alpha 1$ AT genes. Use of oligonucleotide probes to evaluate DNA cloned from a human genomic library known to contain the S $\alpha 1$ AT gene revealed that the two parental $\alpha 1$ AT genes contained in this library were different (Fig. 2). As expected from the study of Long et al. (20), one of the $\alpha 1$ AT genes (λ ATMa17.5) was identified with the four oligonucleotide probes as an S genotype. In this regard, clone λ ATMa17.5 hybridized with the exon III_S probe and exon V_M probe but not the exon III_M probe or the exon V_Z probe, i.e., the sequences in exon III were "S type" and the sequence in the exon V were "M type." Interestingly, however, the other cloned $\alpha 1$ AT gene (λ ATMa14.4) was identified as likely having the M genotype, since it hybridized with the exon III_M probe and the exon V_M probe but not the exon III_S probe or the exon V_Z probe, i.e., the sequences in exon III were "M type" as were the sequences in exon V. Although it is possible that clone λ ATMa14.4 is a rare, non-M haplotype (with "M type" sequences in exon III and V), the haplotype frequency of the M-family in the USA (3–6) makes it likely that the library of Lawn et al. (29) was cloned from an individual who was an $\alpha 1$ AT MS heterozygote.

In addition to clones λ ATMa17.5 (S gene) and λ ATMa14.4 (the probable M gene) from the library of Lawn et al. (29), a cloned $\alpha 1$ AT gene (λ ATM₂) from an individual with the PiM₂M₂ phenotype was examined using the four oligonucleotide probes. Analysis of the autoradiogram patterns showed, as expected, that it was identical to that of λ ATMa14.4, i.e., the sequences in exon III and exon V were "M type" (data not shown). Likewise, evaluation of the $\alpha 1$ AT cDNA clone pTG603 with the

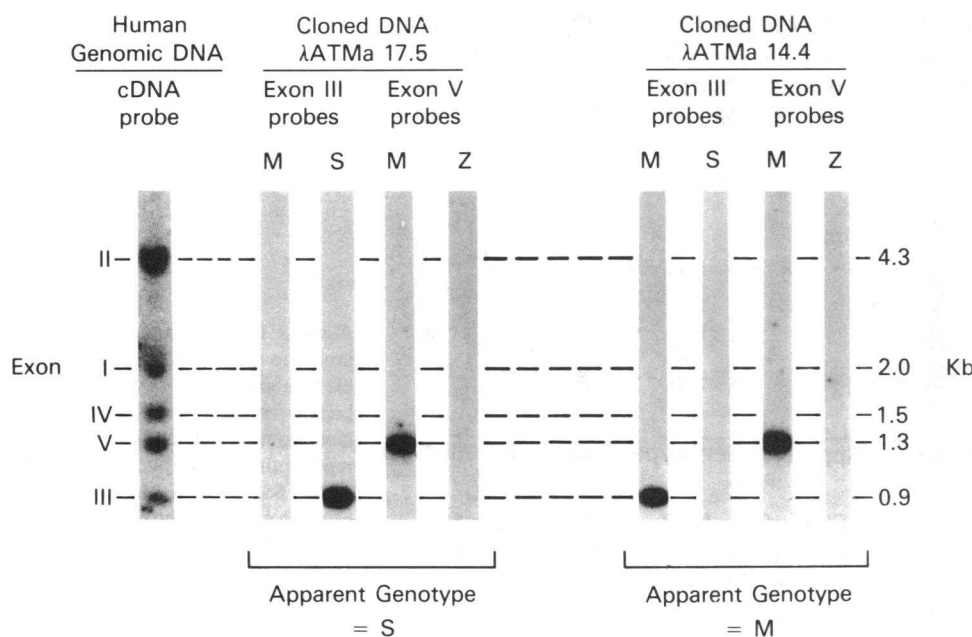


Figure 2. Use of oligonucleotide probes to evaluate the apparent genotype of α 1AT genes cloned from a genomic library known to contain the S type α 1AT gene. Shown are two different clones (designated λ ATMa17.5 and λ ATMa14.4) of the α 1AT gene isolated from the Lawn et al. (29) human genomic library. Both clones contain exons II through V of the α 1AT gene. DNA (1.5 ng/lane) from each clone was digested with restriction endonucleases, BglI and EcoRI, subjected to electrophoresis on 1% agarose gel, followed by blotting on nitrocellulose membranes. Four 32 P-labeled oligonucleotide gene probes, corresponding to exon III_M, exon III_S, exon V_M, and exon V_Z were used for hybridization. After being washed, the nitrocellulose strips were autoradiographed for 3 h at -70°C . As a control, on the left is shown an autoradiogram of human genomic DNA (5 μg) di-

gested with BglI and EcoRI and hybridized with a 32 P-labeled α 1AT cDNA. The sizes of the fragments of genomic DNA containing the five exons are indicated: exon II, 4.3 kb; exon I, 2.0 kb; exon IV, 1.5 kb; exon V, 1.3 kb; and exon III, 0.9 kb. Using the four probes, it is apparent that cloned DNA λ ATMa17.5 has an apparent genotype S and cloned DNA λ ATMa14.4 has an apparent genotype M.

four oligonucleotide probes showed that the sequence in exon III and V were "M type," demonstrating that it was cloned from a library of an individual who carried at least one M gene (data not shown).

These data also demonstrate that, under the conditions used, the synthetic 19-mer oligonucleotide gene probes could adequately discriminate single base differences in the α 1AT gene, including the difference between the S and M genes. Furthermore, using highly specific activity probes and the hybridization conditions described in Methods, this could be accomplished with a clear hybridization signal without significant background.

Correct identification of various combinations of S, M, and Z haplotypes using four oligonucleotide gene probes. Under the conditions used, the four oligonucleotide gene probes could identify single base differences in exon III and V of the α 1AT gene using only 5 μg of total genomic DNA and 2 d of exposure of the autoradiogram (Fig. 3). Furthermore, this was accomplished with hybridization signals that were highly specific without nonspecific background signals.

Using the exon III_S probe, exon III_M probe, exon V_Z probe, and exon V_M probe, the α 1AT phenotypes MM, SS, ZZ, MS, MZ, and SZ (i.e., all possible combinations of the S, M, and Z haplotypes) could be correctly identified at the genomic DNA level (Fig. 3). In this context: (a) The phenotype M₁M₁ yielded positive signals with exon III_M and exon V_M probes but negative signals with the exon III_S and exon V_Z probes. (b) The phenotype SS yielded positive signals with exon III_S and exon V_M probes but negative signals with the exon III_M and exon V_Z probes. (c) The phenotype ZZ yielded positive signals with exon III_M and exon V_Z probes but negative signals with the exon III_S and exon V_M probes. (d) The phenotype M₁S yielded positive signals with exon III_M, exon III_S, and exon V_M probes but a negative signal with exon V_Z probe. (e) The phenotype M₁Z yielded positive signals with exon III_M, exon V_M, and exon V_Z probes but a negative signal with the exon III_S probe. (f) The phenotype SZ

yielded positive signals with all four probes, i.e., the exon III_M, exon III_S, exon V_M, and exon V_Z probes.

It is apparent from this analysis that to correctly identify these six possible combinations of genotype, all four oligonucleotide probes must be used. In this context, if only exon III_M and exon III_S probes are used, the phenotypes MM, SS, and MS would be correctly identified, but the phenotype ZZ would be "misdiagnosed" as "MM", as would the phenotype MZ, and the phenotype SZ would be mistakenly identified as "SM." This analysis also points out that if only exon V_M and exon V_Z probes were used, then the phenotype MM, ZZ, MZ would be correctly identified, but the phenotype SS and MS would be mistakenly identified as "MM" and phenotype SZ would be mistakenly identified as "MZ."

The validity of using the four oligonucleotide probes to correctly identify the MM, SS, ZZ, MS, MZ, and SZ phenotypes at the gene levels was demonstrated by evaluating genomic DNA from 42 individuals with various combinations of the S, Z, and M genes (Table I). All 11 individuals with the serum phenotype MM had positive signals with the exon III_M and exon V_M probes and negative signals with the exon III_S and exon V_Z probes, indicating the apparent genotype to be M, i.e., there was a 100% correlation between serum phenotype and oligonucleotide genotype. Interestingly, the subtype M-haplotypes, M₁, M₂, and M₃, hybridized equally with the exon III_M probe and exon V_M probe, suggesting that 19-base sequences evaluated with the exon III_M and exon V_M probes are identical in the M₁, M₂, and M₃ genes. Thus, whatever the sequence differences among these three M haplotypes, these differences are not present in these two 19-base sequences.

Of the various S-containing phenotypes (i.e., SS, MS, SZ) evaluated, there was a perfect correlation of the genotype identified with the four oligonucleotide probes and the serum phenotypes (Table I). This was also true for the Z, non-S-containing phenotype (i.e., ZZ, MZ). Thus, for the relatively common phe-

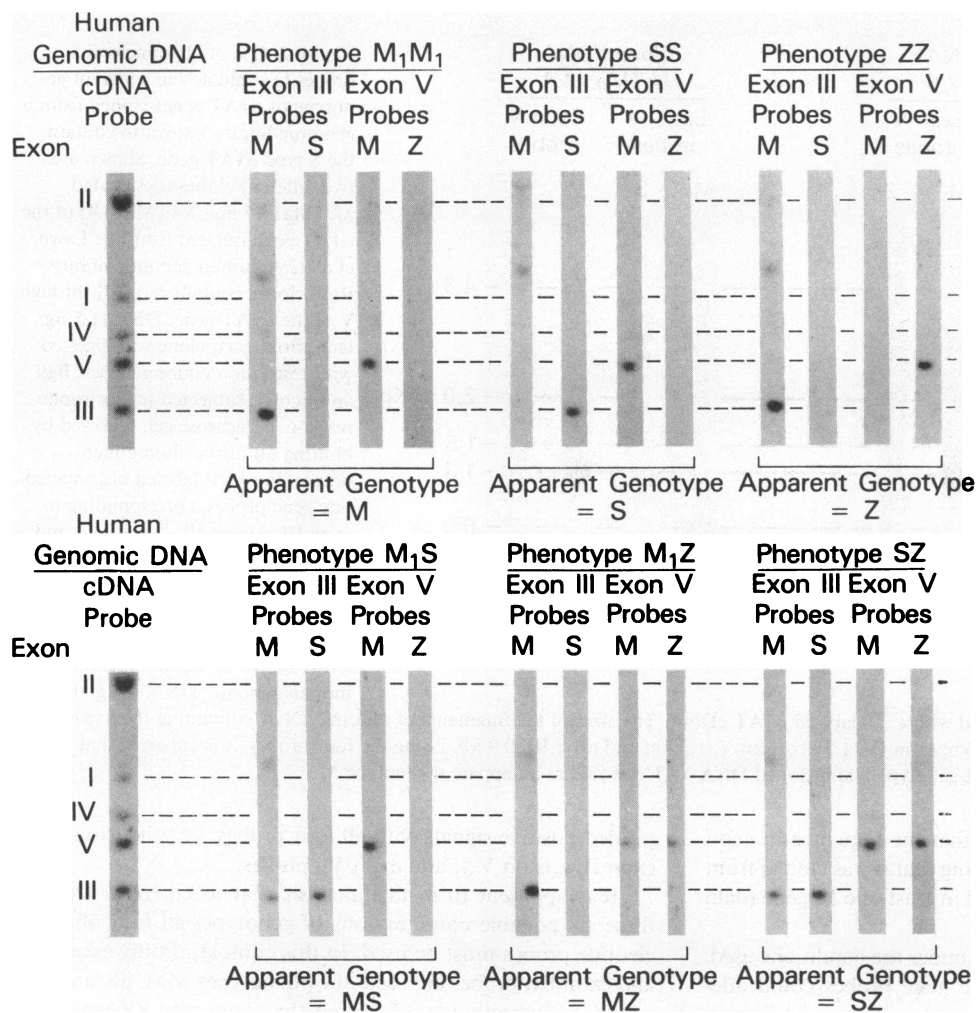


Figure 3. Use of oligonucleotide probes to correctly identify all possible combinations of M, S, and Z haplotypes. Genomic DNA (5 μ g/lane) from six different individuals, representing known phenotypes M_1M_1 , SS, ZZ, M_1S , M_1Z , and SZ, were digested with the endonucleases BglI and EcoRI, electrophoresed on 1% agarose gel, blotted to nitrocellulose filters, and hybridized with 32 P-labeled exon III_M, exon III_S, exon V_M, and exon V_Z probes. After being washed, the nitrocellulose strips were autoradiographed for 2 d at -70°C . As a control, at the left is shown genomic DNA cut with the same endonucleases and hybridized with 32 P-labeled cDNA, thus identifying DNA fragments containing exons I–V (see legends to Figs. 1, 2). The apparent genotype of each sample determined by oligonucleotide probe analysis is below the four electrophoretograms of each phenotype.

notypes that include the S haplotype, the four oligonucleotide probes appear to be sufficient to enable the correct identification of the α 1AT types at the genomic DNA level.

Possible errors in genotypic diagnosis of the S allele using

Table I. Summary of α 1AT Genotype Evaluation of 42 Individuals of Known Serum Phenotypes Using Four Oligonucleotide Gene Probes

Serum phenotype*	n	Number with positive signals for each oligonucleotide probe				Correlation of serum phenotype with oligonucleotide genotype	
		Exon III probes		Exon V probes			
		M	S	M	Z	n	Percent
MM‡	11	11	0	11	0	11/11	100
SS	1	0	1	1	0	1/1	100
ZZ	12	12	0	0	12	12/12	100
MS‡	6	6	6	6	0	6/6	100
MZ‡	8	8	0	8	8	8/8	100
SZ	4	4	4	4	4	4/4	100
Total	42						

* Based on isoelectrofocusing, α 1AT levels, and inheritance pattern.

‡ The individuals with the M haplotypes had various combinations of M1, M2, and M3 as described in Methods.

four oligonucleotide probes. While the four oligonucleotide probes designed to evaluate the S allele yield an accurate assessment of the genotype in most situations, there are rare mutations of the α 1AT gene in which a potential misdiagnosis is possible and could lead to missing an “at risk” genotype. One such example is the “null” mutant, an α 1AT gene that does not code for a detectable α 1AT protein (3). An example of such a situation is the one shown in Fig. 4 in which the null allele has been transmitted for 3 generations. One individual (generation II, individual 4) had the serum phenotype Pi null-null, i.e., had no detectable α 1AT in the serum. This individual mated with an individual with the phenotype Pi M_1S and their progeny (III_1 , III_2) both had the phenotypes Pi S null.

When the entire family was evaluated using the exon III_M, exon III_S, exon V_M, and exon V_Z oligonucleotide probes, it was clear that the S null phenotype was misidentified at the genomic level as “MS” (Fig. 5). Likewise, the phenotype Pi null-null (individual II₄) was misidentified as “M” and the Pi M null phenotypes (I_1 , I_2 , II_1 , and II_2) were also misidentified as “M.” In contrast, the four oligonucleotide probes correctly identified the more common phenotypes MM (II_3) and MS (II_5). Thus, although a very rare circumstance, the four oligonucleotide probes used in the present study were not sufficient to identify the genotype correctly when the null allele is present. Theoretically, the exception to this statement would be the situation in which the mutation causing the null allele was present among the sequences identified by the exon III and exon V probes used, a

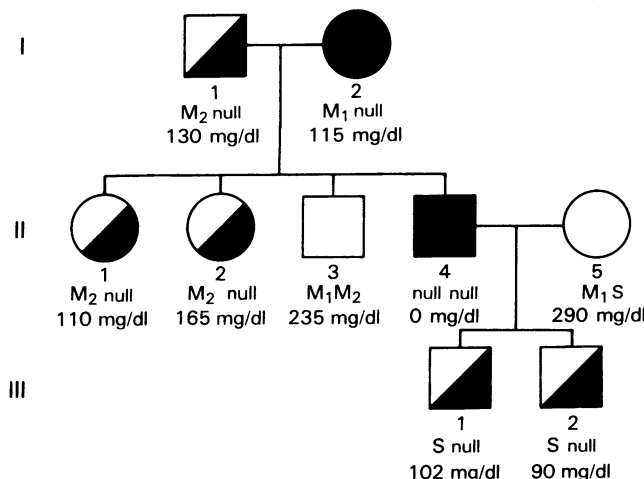


Figure 4. Pedigree of a family with the $\alpha 1$ -antitrypsin null allele. The phenotype of each individual was determined with a combination of isoelectrofocusing on polyacrylamide gel at pH 4 to 5, the serum $\alpha 1$ AT level by immunodiffusion, and analysis of the pedigree. The three generations are indicated (I to III), as are the individual members of each generation (I to *n*). Below each individual ($\square$, males; $\circ$, females) are shown the serum $\alpha 1$ AT phenotype and $\alpha 1$ AT level (mg/dl). The presence of the "null" gene is indicated by shading.

circumstance clearly not the case for the family evaluated in Figs. 4 and 5.

Discussion

The inheritance of the $\alpha 1$ AT S gene in heterozygote combination with the Z gene is associated with an increased risk of the development of adult emphysema and childhood liver disease. Using 19-base length oligonucleotide probes, two to identify the M and S differences in exon III of the $\alpha 1$ AT gene and two to identify the M and Z difference in exon V, we showed in the present study that all six possible combinations of the S, M, and Z $\alpha 1$ AT genes (SS, SZ, MS, MM, ZZ, MZ) can be accurately identified in genomic DNA. These observations demonstrate that using these four oligonucleotide probes, it is possible to accurately identify the "at risk" $\alpha 1$ AT genotype combinations SZ and ZZ in genomic DNA. However, in the circumstance where a very rare "at risk" $\alpha 1$ AT mutant gene (e.g., the null gene) is inherited with the S or Z gene, because the gene sequence mutation may not be localized to the regions of the $\alpha 1$ AT gene covered by the exon III and exon V probes, it is possible to misdiagnose an "at risk" $\alpha 1$ AT genotype combination as "not at risk." However, since the S and Z genes are so much more common than that of any other at risk $\alpha 1$ AT genes, it is reasonable to suggest that using these four oligonucleotide probes, the vast majority of "at risk" $\alpha 1$ AT genotype combinations can be accurately identified in genomic DNA samples, such as those obtained in prenatal studies.

Importance of the S type $\alpha 1$ AT gene. The $\alpha 1$ AT gene is represented by a 10-kb length of chromosome 14 (30). The five exons of this gene code for a mRNA of 1.75 kb in length, a precursor protein of 418 amino acids, and a mature secreted protein of 394 amino acids with three carbohydrate side chains (4, 20). The $\alpha 1$ AT gene is expressed by two classes of cells, hepatocytes and mononuclear phagocytes, with hepatocytes responsible for producing most of the $\alpha 1$ AT (35–38).

The S mutant is among the more than 30 $\alpha 1$ AT haplotypes that have been identified, and along with the Z haplotype represents one of the two major haplotypes that may place the individual at risk for developing disease (3, 5, 6). The most frequent $\alpha 1$ AT haplotypes are those of the M family (M₁, M₂, M₃), together representing at least 90% of all $\alpha 1$ AT haplotypes (6). The S gene is next in frequency; in the USA, the S haplotypic frequency is 0.02–0.04 (8–11) and in Spain and Portugal it is as high as 0.11–0.13 (39, 40). The Z mutant is close behind, representing 0.01–0.02 of all $\alpha 1$ AT haplotypes in the USA (8–11) and in Europe (41).

If inherited with an M-family gene or in the homozygous form, the S mutant does not seem to carry with it any risk for developing lung or liver disease or any other disorder. However, epidemiologic studies from the USA and Europe have shown that when inherited with the Z gene, the S gene clearly confers to the affected individual an increased risk for childhood liver disease (17), adult liver disease (16, 42–44), or emphysema (15, 45–47). There is no question that, for the SZ heterozygote, the S gene contributes to the risk since when the Z gene is inherited with the M gene, there is no increased risk for the development of emphysema (48) and only a questionable risk for the development of liver disease (49).

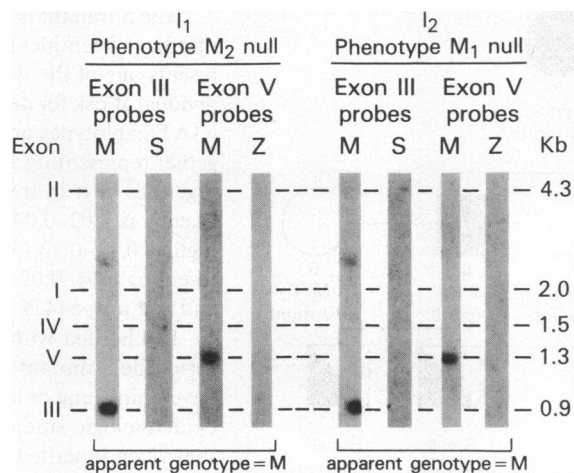
The types of diseases associated with the SZ heterozygote state are similar to that associated with the ZZ homozygote state. The liver disease includes neonatal hepatitis (17), adult cirrhosis (16, 42, 43) and hepatocellular carcinoma (44). The emphysema usually develops by age 40 yr and is characterized by destruction of the lung parenchyma, first developing in the lower lobes, and it is probably accelerated by cigarette smoking (45, 46, 50).

Although the ZZ and SZ states have not been compared extensively, the fact that the frequency of the S gene is greater than that of the Z gene, yet there are fewer cases of liver and/or lung disease reported in association with the SZ state than the ZZ state, suggests that while the S gene carries a clear risk, it is less than that associated with the Z gene. The mechanism by which the S gene is associated with liver disease is unknown. For the lung disease, however, it is assumed that the emphysema associated with the SZ state relates to the fact that these individuals have serum levels thought to be in the range just at or below the threshold level needed by the lung to handle its burden of neutrophil elastase (2, 51). This concept is consistent with the knowledge that the frequency of emphysema is more common among ZZ individuals (serum levels always <50 mg/dl) than SZ individuals (45–105 mg/dl) (51).

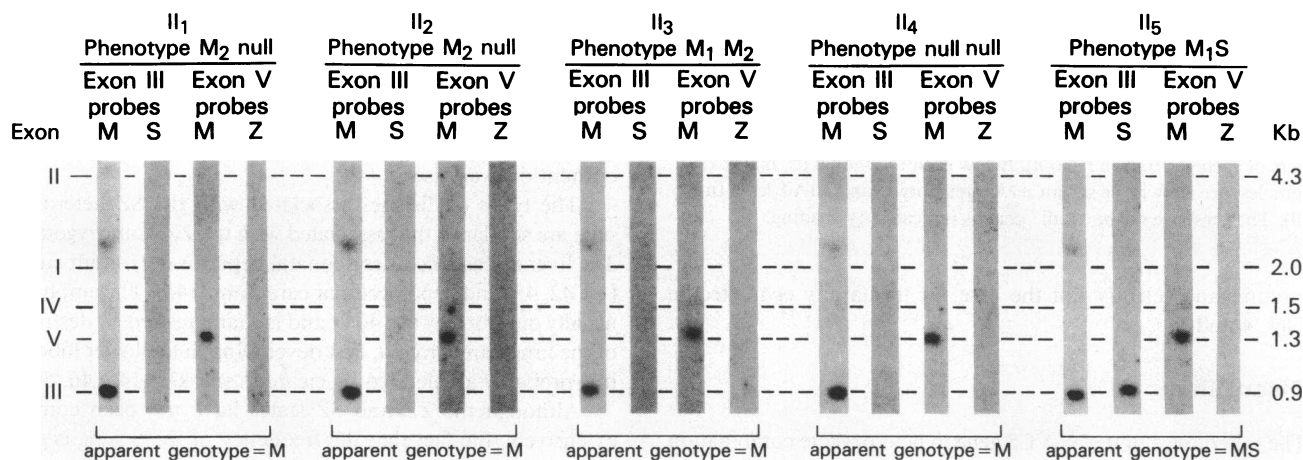
The reason why the S gene is associated with decreased serum levels of $\alpha 1$ AT is unknown. It is not known if the S gene is transcribed normally, if the S mRNA is stable, or if the S protein functions normally. However, it is reasonable to hypothesize that, like the Z protein, the S protein may get trapped in the rough endoplasmic reticulum because it folds into its tertiary structure more slowly than normal, permitting the S molecules to aggregate through hydrophobic interactions (52) or, alternatively, placing the S molecules at risk for being degraded within the cell.

Use of oligonucleotide probes to detect single base changes in the $\alpha 1$ AT gene. The oligonucleotide probe method of genotypic diagnosis is based on the concept that, under carefully defined conditions, a short (usually 19-mer) segment of DNA will hybridize to genomic DNA only if the sequence of the oligonucleotide is completely complementary to a sequence in genomic DNA. Originally developed by Wallace et al. (53, 54),

I



II



III

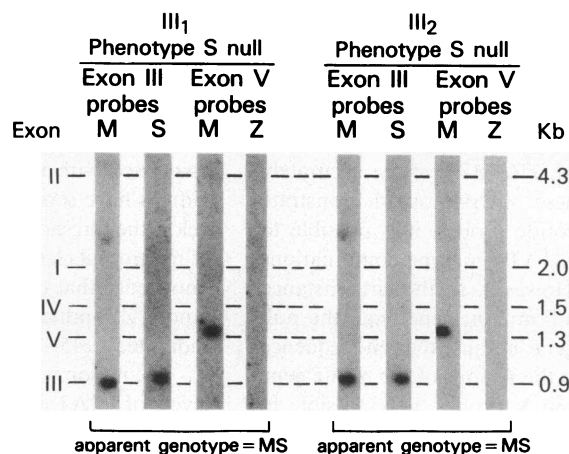


Figure 5. Evaluation of a family with the $\alpha 1$ -antitrypsin null allele using oligonucleotide probes designed to detect exon III M and S gene sequences and exon V, M, and Z gene sequences. Total genomic DNA (5 μ g/lane) of each family member was digested with the endonucleases BglI and EcoRI, electrophoresed on 1% agarose gel, and blotted to nitrocellulose. The blotted DNA was then hybridized with 32 P-labeled exon III_M, exon III_S, exon V_M, and exon V_Z probes, washed, and autoradiogrammed (2 d, -70°C). Dotted lines indicate sizes of DNA

fragments containing exon II (4.3 kb), exon I (2.0 kb), exon IV (1.5 kb), exon V (1.3 kb), and exon III (0.9 kb). The phenotype as determined by serum analysis is indicated above the analysis of each family member and the apparent genotype as determined with the four oligonucleotide probes is indicated below. The grouping of the analysis of the pedigree is displayed in the same pattern as shown in Fig. 4, with each family member indicated by a corresponding generation number (I to III) and individual member (1 to *n*).

the oligonucleotide gene probe method was designed with the theoretical background that synthetic oligonucleotides of 19 residues in length have a high probability of recognizing a unique sequence in the human genome (55). The oligonucleotide

method has been shown to identify single-point mutation at the genomic DNA level for the $\alpha 1$ AT gene (22, 23), the β -globin gene (56–61), and the H-ras gene (62).

Sufficient experience has been gained with this approach to

genotype diagnosis to validate that the method clearly can distinguish among genomic DNAs with sequences that are identical except for 1-base. One limitation of the method is the need for high specific activity labeling of the probes (58). To overcome this problem, instead of using 5' labeling, we labeled the 19-mer oligonucleotide probes using primer extension (32), resulting in specific activities as high as 10^8 dpm/pmol (i.e., about the same specific activity as nick translated DNA probes). Furthermore, although hybridization on dried agarose gels has been reported to be more sensitive than hybridization to DNA transferred to nitrocellulose membranes (32), we have been able to obtain satisfactory blotting signals using nitrocellulose membranes without nonspecific hybridization signals to large DNA fragments.

Although oligonucleotide probes can accurately distinguish point mutations, their use assumes that: (a) all point mutations relevant to the gene in question are known; (b) a sufficient number of probes are used to account for all of the relevant point mutations; and (c) no unknown mutations (single base or otherwise) occur in the 19 bases complementary to the probes being used (independent of whether they code for different amino acids or not). These concepts are particularly important if clinical decisions are to be based on the information derived, such as prenatal diagnosis to help in genetic counseling. For the $\alpha 1$ AT gene, besides the common M-family haplotypes there are more than 30 haplotypes known, but the molecular basis is known for only two common haplotypes, the Z and S genes, and one rare haplotype ($M_{\text{Pittsburg}}$, met³⁵⁸ to arg³⁵⁸) (63). However, of all the known mutations that do put the affected individual at risk for the development of disease, only the Z and S haplotypes are found in the population in sufficient numbers to be relevant for general diagnostic purposes.

It is clear from the present study, however, that to accurately identify the relatively common at risk phenotypes SZ and ZZ, it is critical to use four probes, two designed to evaluate exon V (the site of the Z mutation) and two designed to evaluate exon III (the site of the S mutation). With this approach it is possible to correctly identify the six phenotypes of the possible combinations of S, M, and Z haplotypes. Since these three haplotypes probably cover >98% of the possible occurring $\alpha 1$ AT phenotypes (8) and the vast majority of the at risk phenotypes (3), it is reasonable to suggest that such an approach could be used with reasonable certainty for prenatal genotype identification. However, it is also clear that, using only probes for exon V (22, 23), the relatively frequent S mutation would be scored as "normal" and thus the heterozygote SZ might be misdiagnosed as MZ, a misdiagnosis that may be very relevant in genetic counseling.

Although the combined use of exon III and exon V probes markedly increases the accuracy of diagnosis of "at risk" $\alpha 1$ AT phenotypes, keep in mind that under extraordinarily rare circumstances it may also lead to incorrect genotype diagnosis with potentially serious consequences. For example, in the family evaluated in which the "null" gene was present, even the use of two exon III and two exon V probes misidentified the M null individuals (family members I₁, I₂, II₁, II₂) as genotype "M." Furthermore, since the serum $\alpha 1$ AT levels of these individuals were 110–165 mg/dl, without the pedigree analysis, even the combination of isoelectric focusing, serum $\alpha 1$ AT levels, and oligonucleotide probe analysis would likely lead to a misdiagnosis, an obvious problem in genetic counseling. Perhaps more importantly, using oligonucleotide analysis, the null-null individual (II₄) would be misidentified as genotype "M"; a critical error in the situation of prenatal diagnosis in which serum was not avail-

able. Furthermore, the offspring of this individual would be misdiagnosed as MS, although both (III₁, III₂) were actually phenotype S null. Since the MS phenotype is not a risk, while the S null phenotype will almost certainly develop disease, this could result in a critical error in prenatal diagnosis.

In summary, in view of the frequency of the S haplotype in the general population, and the known association of the SZ phenotype with the risk for disease, it is clear that the use of oligonucleotide probes for $\alpha 1$ AT genotype diagnosis cannot depend on probes designed to identify only the Z mutation. In contrast, if probes are used to evaluate the S and Z mutations (i.e., exon III and V, respectively), almost all "at risk" $\alpha 1$ AT genotypes will be accurately identified. Even so, the known existence of the null haplotype, as well as a few other very rare haplotypes, such as Pi^P (64), Pi^MDuarte (65), and Pi^MMalton (66), demand that the application of this methodology to prenatal diagnosis and genetic counseling must always be used with caution and, whenever possible, in conjunction with pedigree studies, $\alpha 1$ AT serum levels, and isoelectric focusing.

References

1. Travis, J., and G. S. Salvesen. 1983. Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* 52:655–709.
2. Gadek, J. E., G. W. Hunninghake, G. A. Fells, R. L. Zimmerman, B. A. Keogh, and R. G. Crystal. 1980. Evaluation of the protease-antiprotease theory of human destructive lung disease. *Bull. Europ. Physio-pathol. Respir.* 16(Suppl):27–40.
3. Gadek, J. E., and R. G. Crystal. 1982. $\alpha 1$ -Antitrypsin deficiency. In *The Metabolic Basis of Inherited Disease*. 5th ed. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, editors. McGraw-Hill, Inc., New York. 1450–1467.
4. Carrell, R. W., J.-O. Jeppsson, C.-B. Laurell, S. O. Brennan, M. C. Owen, L. Vaughan, and D. R. Boswell. 1982. Structure and variation of human $\alpha 1$ -antitrypsin. *Nature (Lond.)* 298:329–334.
5. Carrell, R. W., and M. C. Owen. 1979. $\alpha 1$ -antitrypsin structure, variation and disease. *Essays Med. Biochem.* 4:83–119.
6. Fagerhol, M. K., and D. W. Cox. 1981. The Pi polymorphism: genetic, biochemical, and clinical aspects of human $\alpha 1$ -antitrypsin. *Adv. Hum. Genet.* 11:1–62.
7. Kueppers, F. 1978. Inherited differences in alpha 1-antitrypsin. In *Genetic Determinants of Pulmonary Disease*. S. D. Litwin, editor. Marcel Dekker, Inc., New York. 23–74.
8. Pierce, J. A., B. Eradio, and T. A. Dew. 1975. Antitrypsin phenotypes in St. Louis. *J. Am. Med. Assoc.* 231:609–612.
9. Dykes, D. D., S. A. Miller, and H. F. Polesky. 1984. Distribution of $\alpha 1$ -antitrypsin variations in a US white population. *Hum. Hered.* 34:308–310.
10. Evans, H. E., N. S. Bognacki, L. M. Perrott, and L. Glass. 1977. Prevalence of alpha 1-antitrypsin Pi types among newborn infants of different ethnic backgrounds. *J. Pediatr.* 90:621–624.
11. Kueppers, F., and M. J. Christopherson. 1978. Alpha₁-antitrypsin: further genetic heterogeneity revealed by isoelectrofocusing. *Am. J. Hum. Genet.* 30:359–365.
12. Laurell, C.-B., and S. Eriksson. 1963. The electrophoretic $\alpha 1$ -globulin pattern of serum in $\alpha 1$ -antitrypsin deficiency. *Scand. J. Clin. Lab. Invest.* 15:132–140.
13. Eriksson, S. 1965. Studies in $\alpha 1$ -antitrypsin deficiency. *Acta Med. Scand.* 177(432):6–85.
14. Morse, J. O. 1978. Alpha₁-antitrypsin deficiency. *N. Engl. J. Med.* 299:1045–1048 and 1099–1105.
15. Larsson, C., H. Dirksen, G. Sundstrom, and S. Eriksson. 1976. Lung function studies in asymptomatic individuals with moderately (Pi SZ) and severely (Pi Z) reduced levels of $\alpha 1$ -antitrypsin. *Scand. J. Respir. Dis.* 57:267–280.
16. Craig, J. R., A. E. G. Dunn, and R. L. Peters. 1975. Cirrhosis

associated with partial deficiency of alpha-1-antitrypsin: a clinical and autopsy study. *Hum. Pathol.* 6:113-120.

17. Sveger, T. 1976. Liver disease in alpha₁-antitrypsin deficiency detected by screening of 200,000 infants. *N. Engl. J. Med.* 294:1316-1321.

18. Yoshida, A., J. Lieberman, L. Gaidulis, and C. Ewing. 1976. Molecular abnormality of human alpha₁-antitrypsin variant (Pi-ZZ) associated with plasma activity deficiency. *Proc. Natl. Acad. Sci. USA.* 73:1324-1328.

19. Yoshida, A., C. Ewing, M. Wessels, J. Lieberman, and L. Gaidulis. 1977. Molecular abnormality of PiS variant of human alpha₁-antitrypsin. *Am. J. Hum. Genet.* 29:233-239.

20. Long, G. L., T. Chandra, S. L. C. Woo, E. W. Davie, and K. Kurachi. 1984. Complete sequence of the cDNA for human alpha₁-antitrypsin and the gene for the S variant. *Biochemistry.* 23:4828-4837.

21. Cox, D. W., A. M. Johnson, and M. K. Fagerhol. 1980. Report of nomenclature meeting for alpha₁-antitrypsin: INSERM, Rouen/Bois-Guillaume 1978. *Hum. Genet.* 53:429-433.

22. Kidd, V. J., R. B. Wallace, K. Itakura, and S. L. C. Woo. 1983. alpha₁-Antitrypsin deficiency detection by direct analysis of the mutation in the gene. *Nature (Lond.)*. 304:230-234.

23. Kidd, V. J., M. S. Golbus, R. B. Wallace, K. Itakura, and S. L. C. Woo. 1984. Prenatal diagnosis of alpha₁-antitrypsin deficiency by direct analysis of the mutated site in the gene. *N. Engl. J. Med.* 310:639-642.

24. Garver, R., J. Mornex, T. Nukiwa, M. Brantly, M. Courtney, J. LeCocq, and R. Crystal. 1986. alpha₁-antitrypsin deficiency and emphysema caused by homozygous inheritance of non-expressing alpha₁-antitrypsin genes. *N. Engl. J. Med.* In press.

25. Frants, R. R., G. T. Noordhoek, and A. W. Eriksson. 1978. Separator isoelectric focusing for identification of alpha₁-antitrypsin (PiM) subtypes. *Scand. J. Clin. Lab. Invest.* 38:457-462.

26. Constans, J., M. Viau, and C. Gouaillard. 1980. Pi^{M4}: an additional Pi^M subtype. *Hum. Genet.* 55:119-121.

27. Jeppsson, J.-O., C.-B. Laurell, and M. Fagerhol. 1978. Properties of isolated human alpha₁-antitrypsin of Pi types M, S and Z. *Eur. J. Biochem.* 83:143-153.

28. Jeffreys, A. J., and R. A. Flavell. 1977. A physical map of the DNA regions flanking the rabbit beta-globin gene. *Cell.* 12:429-439.

29. Lawn, R. M., E. F. Fritsch, R. C. Parker, G. Blake, and T. Maniatis. 1978. The isolation and characterization of linked delta- and beta-globin genes from a cloned library of human DNA. *Cell.* 15:1157-1174.

30. Courtney, M., A. Buchwalder, L.-H. Tessier, M. Jaye, A. Benavente, A. Balland, V. Kohli, R. Lathe, P. Tolstoshev, and J.-P. LeCocq. 1984. High-level production of biologically active human alpha₁-antitrypsin in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* 81:669-673.

31. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York. pp. 76-85.

32. Studencki, A. B., and R. B. Wallace. 1984. Allele-specific hybridization using oligonucleotide probes of very high specific activity: Discrimination of the human beta_A- and beta_S-globin genes. *DNA (NY)*. 3:7-15.

33. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.

34. Lai, E. C., F. T. Kao, M. L. Law, and S. L. C. Woo. 1983. Assignment of the alpha₁-antitrypsin gene and a sequence-related gene to human chromosome 14 by molecular hybridization. *Am. J. Hum. Genet.* 35:385-392.

35. Geiger, T., W. Northemann, E. Schmelzer, V. Gross, F. Gauthier, and P. C. Heinrich. 1982. Synthesis of alpha₁-antitrypsin in rat-liver hepatocytes and in a cell-free system. *Eur. J. Biochem.* 126:189-195.

36. Ciliberto, G., L. Dente, and R. Cortese. 1985. Cell-specific expression of a transfected human alpha₁-antitrypsin gene. *Cell.* 41:531-540.

37. Perlmutter, D. H., F. S. Cole, P. Kilbridge, T. H. Rossing, and H. R. Colten. 1985. Expression of the alpha₁-proteinase inhibitor gene in

human monocytes and macrophages. *Proc. Natl. Acad. Sci. USA.* 82:795-799.

38. Mornex, J.-F., M. Miskulin, R. Dagleish, M. Courtney, P. Tolstoshev, J.-P. LeCocq, and R. G. Crystal. 1984. Expression of the alpha₁-antitrypsin gene in human blood monocytes and alveolar macrophages. *Clin. Res.* 32(2):433a(Abstr.)

39. Goedde, H. W., L. Hirht, H.-G. Benkmann, A. Pellicer, T. Pellicer, M. Stehn, and S. Singh. 1973. Population genetic studies of serum protein polymorphisms in four Spanish populations. II. *Hum. Hered.* 23:135-146.

40. Martin, J.-P., D. Vandeville, C. Ropartz, and M. T. Pereira. 1976. Genetic variations of serum alpha₁-antitrypsin (Pi types) in Portuguese. *Hum. Hered.* 26:310-314.

41. Fagerhol, M. K. 1967. Serum Pi types in Norwegians. *Acta Pathol. Microbiol. Scand.* 72:421-428.

42. Campa, J. L., J. R. Craig, R. L. Peters, and T. B. Reynolds. 1973. Cirrhosis associated with partial deficiency of alpha₁-antitrypsin in an adult. *Ann. Intern. Med.* 78:233-238.

43. Palmer, P. E., G. J. Gherardi, J. M. Baldwin, and H. J. Wolfe. 1978. Adult liver disease in SZ phenotype alpha₁-antitrypsin deficiency. *Ann. Intern. Med.* 88:59-60.

44. Chan, C. H., C. J. Steer, J. Vergalla, and E. A. Jones. 1978. Alpha₁-antitrypsin deficiency with cirrhosis associated with the protease inhibitor phenotype SZ. *Am. J. Med.* 65:978-986.

45. Hutchison, D. C. S., M. J. Tobin, and P. J. L. Cook. 1983. Alpha₁-antitrypsin deficiency: Clinical and physiological features in heterozygotes of Pi type SZ: A survey by the British Thoracic Association. *Br. J. Dis. Chest.* 77:28-34.

46. Gishen, P., A. J. S. Saunders, M. J. Tobin, and D. C. S. Hutchison. 1982. Alpha₁-antitrypsin deficiency: the radiological features of pulmonary emphysema in subjects of Pi type Z and Pi type SZ: a survey by the British thoracic association. *Clin. Radiol.* 33:371-377.

47. Morse, J. O., M. D. Lebowitz, R. J. Knudson, and B. Burrows. 1977. Relation of protease inhibitor phenotypes to obstructive lung diseases in a community. *N. Engl. J. Med.* 296:1190-1194.

48. Bruce, R. M., B. H. Cohen, E. L. Diamond, J. Fallat, R. J. Knudson, M. D. Lebowitz, C. Mittman, C. D. Patterson, and M. S. Tockman. 1984. Collaborative study to assess risk of lung disease in PiMZ phenotype subjects. *Am. Rev. Respir. Dis.* 130:386-390.

49. Fisher, R. L., L. Taylor, and S. Sherlock. 1976. alpha₁-antitrypsin deficiency in liver disease: the extent of the problem. *Gastroenterology.* 71:646-651.

50. Hutchison, D. C. S. 1980. A survey of alpha₁-antitrypsin deficiency by the British thoracic association. *Bull. Europ. Physiopathol. Respir.* 16(Suppl.):315-319.

51. Gadek, J. E., H. G. Klein, P. V. Holland, and R. G. Crystal. 1981. Replacement therapy of alpha₁-antitrypsin deficiency: reversal of protease-antiprotease imbalance within the alveolar structures of PiZ subjects. *J. Clin. Invest.* 68:1158-1165.

52. Loebermann, H., R. Tokunaka, J. Deisenhofer, and R. Huber. 1984. Human alpha₁-proteinase inhibitor: crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J. Mol. Biol.* 177:531-556.

53. Wallace, R. B., M. Schold, M. J. Johnson, P. Dembek, and K. Itakura. 1981. Oligonucleotide directed mutagenesis of the human beta-globin gene: a general method for producing specific point mutations in cloned DNA. *Nucleic Acids Res.* 9:3647-3656.

54. Itakura, K., J. J. Rossi, and R. B. Wallace. 1984. Synthesis and use of synthetic oligonucleotides. *Annu. Rev. Biochem.* 53:323-356.

55. Nei, M., and W.-H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA.* 76:5269-5273.

56. Conner, B. J., A. A. Reyes, C. Morin, K. Itakura, R. L. Teplitz, and R. B. Wallace. 1983. Detection of sickle cell betaS-globin allele by hybridization with synthetic oligonucleotides. *Proc. Natl. Acad. Sci. USA.* 80:278-282.

57. Pirastu, M., Y. W. Kan, A. Cao, B. J. Conner, R. L. Teplitz, and

- R. B. Wallace. 1983. Prenatal diagnosis of β -thalassemia: detection of a single nucleotide mutation in DNA. *N. Engl. J. Med.* 309:284–287.
58. Orkin, S. H., A. F. Markham, and H. H. Kazazian. 1983. Direct detection of the common Mediterranean β -thalassemia gene with synthetic DNA probes: an alternative approach for prenatal diagnosis. *J. Clin. Invest.* 71:775–779.
59. Kazazian, H. H., Jr., S. H. Orkin, A. F. Markham, C. R. Chapman, H. Youssoufian, and P. G. Waber. 1984. Quantification of the close association between DNA haplotypes and specific β -thalassaemia mutations in Mediterraneans. *Nature (Lond.)*. 310:152–154.
60. Rosatelli, C., A. M. Falchi, T. Tuveri, M. T. Scalas, A. Di Tucci, G. Monni, and A. Cao. 1985. Prenatal diagnosis of beta-thalassaemia with the synthetic-oligomer technique. *Lancet*. i:241–243.
61. Studencki, A., B. J. Conner, C. C. Impraim, R. L. Teplitz, and R. B. Wallace. 1985. Discrimination among the human β^A , β^S , and β^C -globin genes using allele-specific oligonucleotide hybridization probes. *Am. J. Hum. Genet.* 37:42–51.
62. Bos, J. L., D. Toksoz, C. J. Marshall, M. Verlaan-de Vries, G. H. Veeneman, A. J. van der Eb, J. H. van Boom, J. W. G. Janssen, and A. C. M. Steenvoorden. 1985. Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukaemia. *Nature (Lond.)*. 315:726–730.
63. Owen, M. C., S. O. Brennan, J. H. Lewis, and R. W. Carrell. 1983. Mutation of α 1AT to α 1AT Pittsburg (³⁵⁸met lead to arg), a fatal bleeding disorder. *N. Engl. J. Med.* 309:694–698.
64. Fagerhol, M. K., and H. E. Hauge. 1969. Serum Pi types in patients with pulmonary diseases. *Acta Allergol.* 24:107–114.
65. Lieberman, J., L. Gaidulis, and S. D. Klotz. 1976. A new deficient variant of α 1-antitrypsin (^MDuarte): inability to detect the heterozygous state by antitrypsin phenotyping. *Am. Rev. Respir. Dis.* 113:31–36.
66. Sproule, B. J., D. W. Cox, K. Hsu, M. L. Salkie, and F. A. Herbert. 1983. Pulmonary function associated with the ^MMalton deficient variant of α 1-antitrypsin. *Am. Rev. Respir. Dis.* 127:237–240.