

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

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## Abstract

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  $^{32}$ 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"  $\alpha$ 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 at risk" MM and SM combinations. These observations indicate that oligonucleotide gene probes yielded reliable and accurate assessment of "at risk"  $\alpha$ 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 ( $\alpha$ 1AT)<sup>1</sup>, 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  $\alpha$ 1AT gene is highly pleomorphic; more than 30 different haplotypes have been described (3, 6). The  $\alpha$ 1AT phenotype, referred to as the protease inhibitor (Pi) type, represents the codominant expression of the two parental  $\alpha$ 1AT haplotypes (3).

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1. *Abbreviations used in this paper:*  $\alpha$ 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.

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The most common  $\alpha$ 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  $\alpha$ 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 val<sup>264</sup> replaces glu<sup>264</sup>, while in the Z protein lys<sup>342</sup> replaces glu<sup>342</sup> (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  $\alpha$ 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  $\alpha$ 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  $\alpha$ 1AT gene to include the S gene such that all possible "at risk"  $\alpha$ 1AT phenotypes involving the S, Z, and M genes can be accurately identified. By evaluating 42 individuals with a variety of known  $\alpha$ 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  $\alpha$ 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  $\alpha$ 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:  $M_1M_2$  ( $n = 1$ );  $M_{null}$  ( $n = 4$ ;  $M_{1null} = 1$ ,  $M_{2null} = 3$ );  $M_1S$  ( $n = 1$ ); and  $S_{null}$  ( $n = 2$ ).

**Determination of  $\alpha$ 1AT phenotype.**  $\alpha$ 1AT phenotypes (referred to as the "Pi" type) were determined by a combination of isoelectrofocusing, serum  $\alpha$ 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  $\alpha$ 1AT M subtypes using a modification of the method of Constans et al. (25, 26).  $\alpha$ 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  $\alpha$ 1AT standards used by clinical laboratories overestimate  $\alpha$ 1AT serum levels by ~35%. Assignment of  $\alpha$ 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  $-70^\circ\text{C}$ . 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  $\alpha$ 1AT genes.** Three human  $\alpha$ 1AT genomic clones were used in this study, including: (a)  $\lambda$ 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)  $\lambda$ ATMa17.5, a 17.5-kb fragment of the Lawn et al. library; and (c)  $\lambda$ ATM<sub>2</sub>, a 10-kb fragment of a human DNA  $\lambda$ WES lambda phage library constructed from an individual known to have  $\alpha$ 1AT PiM<sub>2</sub>M<sub>2</sub>. All clones were identified using a full-length  $\alpha$ 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  $^{32}\text{P}$  by nick translation (Amersham Corp., Arlington Heights, IL) and used to screen the genomic DNA libraries.

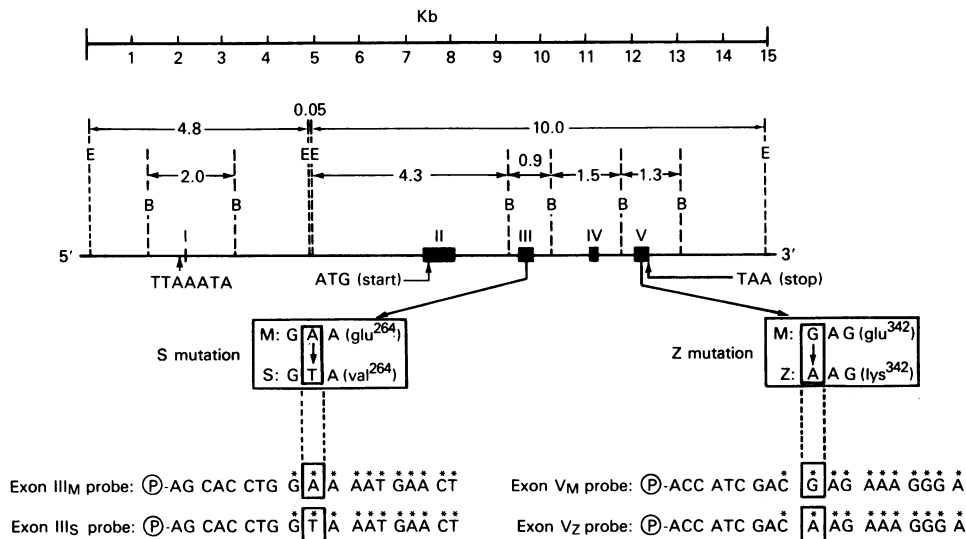
From the studies of Long et al. (20) it is known that the human genomic DNA library constructed by Lawn et al. (29) contains the S  $\alpha$ 1AT gene. Thus, to provide a control for the use of oligonucleotide probes to evaluate the S  $\alpha$ 1AT gene, the Lawn et al. (29) library was screened for fragments containing the  $\alpha$ 1AT gene. This was accomplished by evaluating  $10^6$  plaque-forming unit of the library, using the  $\alpha$ 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,  $\lambda$ 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  $\alpha$ 1AT gene; this clone was designated  $\lambda$ ATMa17.5. The phage containing  $\alpha$ 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  $65^\circ\text{C}$ , 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  $\alpha$ 1AT serum phenotype Pi M<sub>2</sub>M<sub>2</sub> (i.e., homozygous for the M<sub>2</sub>  $\alpha$ 1AT gene; isoelectrofocusing Pi type M<sub>2</sub>, serum  $\alpha$ 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  $\mu\text{g}$ ) was completely digested with the restriction endonuclease EcoRI (1 U/ $\mu\text{g}$  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  $\alpha$ 1AT gene (see map, Fig. 1) were detected by hybridization with  $^{32}\text{P}$ -labeled  $\alpha$ 1AT cDNA as a probe. The DNA fraction (0.5  $\mu\text{g}$ ) containing the 10.0 kb EcoRI fragment was packaged in vitro into  $\lambda$ gtWES phage using a kit supplied by Amersham Corp. Starting from  $10^5$  plaque-forming unit lambda phages, one clone was isolated using  $^{32}\text{P}$ -labeled  $\alpha$ 1AT cDNA as a probe. This clone, designated  $\lambda$ ATM<sub>2</sub>, was demonstrated by restriction mapping with  $^{32}\text{P}$ -labeled cDNA to contain the 10.0-kb EcoRI insert of the  $\alpha$ 1AT gene containing exons II to V.

**Preparation of  $^{32}\text{P}$ -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). [ $\alpha$ - $^{32}\text{P}$ ]dATP (6,000 Ci/mmol, 250  $\mu\text{Ci}$ , 36 pmol), [ $\alpha$ - $^{32}\text{P}$ ]dCTP (3,000 Ci/mmol, 125  $\mu\text{Ci}$ , 42 pmol), [ $\alpha$ - $^{32}\text{P}$ ]dGTP (3,000 Ci/mmol, 125  $\mu\text{Ci}$ , 42 pmol), and [ $\alpha$ - $^{32}\text{P}$ ]TTP (3,000 Ci/mmol, 125  $\mu\text{Ci}$ , 42 pmol) were dried, and a mixture of primer extension reaction reagents (50 mM NaCl, 25 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 0.72  $\mu\text{M}$  template [19-mer], 2.7  $\mu\text{M}$  primer [phosphorylated 8-mer], and 2.3 units *E. coli* DNA polymerase I [Klenow fragment]) was added to a final volume of 5  $\mu\text{l}$ . The reaction was performed at  $4^\circ\text{C}$  for 3 h and terminated with the addition of 5  $\mu\text{l}$  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  $^{32}\text{P}$ -labeled oligonucleotide probes was stored at  $-20^\circ\text{C}$ . 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;  $2 \times 10^{10}$  dpm/ $\mu\text{g}$ ).

**Strategy for evaluation of S mutation using oligonucleotide gene probes.** The strategy used for the evaluation of the S mutation of the  $\alpha$ 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 BglI; together these endonucleases cut the  $\alpha$ 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 BglI-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 glu<sup>264</sup> to be altered to val<sup>264</sup>. The 19-mer oligonucleotide gene probes used to detect this change are indicated as "exon III<sub>M</sub> probe" and "exon III<sub>S</sub> 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 <sup>32</sup>P (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 glu<sup>342</sup> to be altered to lys<sup>342</sup>. Shown are the sequences of the oligonucleotide gene probes used to detect these sequences "exon V<sub>M</sub> probe" and "exon V<sub>Z</sub> 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<sub>M</sub> probe" and "exon III<sub>S</sub> 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 glu<sup>264</sup> to val<sup>264</sup> in the  $\alpha 1$ AT protein. Two probes (referred to as "exon V<sub>M</sub> probe" and "exon V<sub>Z</sub> 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 glu<sup>342</sup> to lys<sup>342</sup> in the  $\alpha 1$ AT protein.

**Hybridization and autoradiography using oligonucleotide probes.** Human genomic DNA (20–50  $\mu$ g) was digested with restriction endonuclease BglII (1 U/ $\mu$ g of DNA) for 8 h, 37°C, and then endonuclease EcoRI (1 U/ $\mu$ 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  $\mu$ 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.

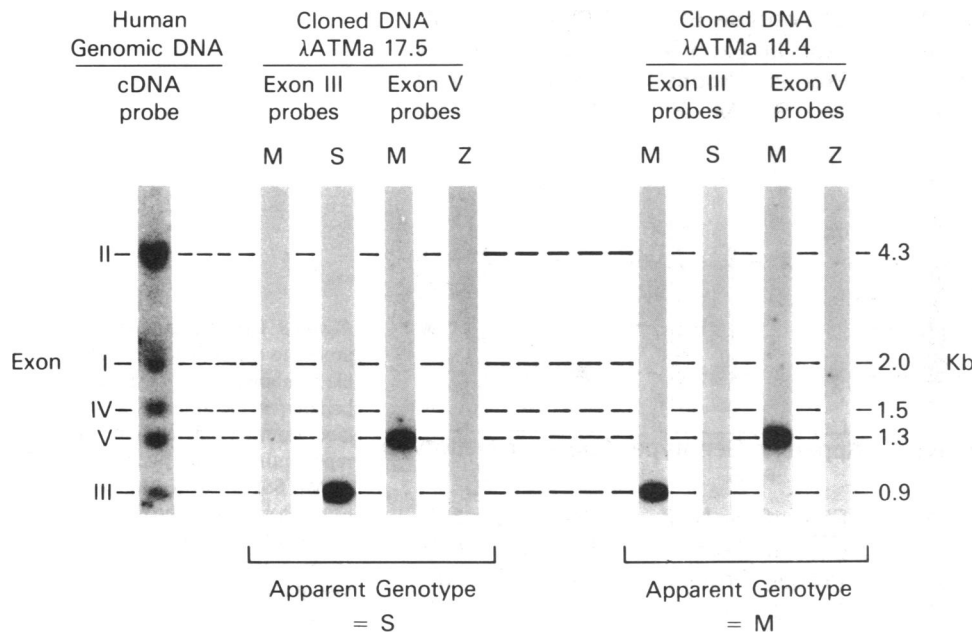
Before hybridization, blotted and baked nitrocellulose sheets were exposed to 100 ml of 6 $\times$  SSC (1 $\times$  SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 $\times$  Denhardt's solution (1 $\times$  Denhardt's solution = 0.2 g Ficoll, 0.2 g polyvinylpyrrolidone, and 0.2 g bovine serum albumin [Pentax Fraction V] in 1 liter H<sub>2</sub>O), 0.1% sodium dodecyl sulfate, 0.05% sodium pyrophosphate at 65°C for 4 h. Hybridization with the <sup>32</sup>P-labeled 19-mer oligonucleotide (10<sup>6</sup> 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  $-70^{\circ}\text{C}$  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 ( $\lambda$ ATMa17.5) was identified with the four oligonucleotide probes as an S genotype. In this regard, clone  $\lambda$ ATMa17.5 hybridized with the exon III<sub>S</sub> probe and exon V<sub>M</sub> probe but not the exon III<sub>M</sub> probe or the exon V<sub>Z</sub> 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 ( $\lambda$ ATMa14.4) was identified as likely having the M genotype, since it hybridized with the exon III<sub>M</sub> probe and the exon V<sub>M</sub> probe but not the exon III<sub>S</sub> probe or the exon V<sub>Z</sub> probe, i.e., the sequences in exon III were "M type" as were the sequences in exon V. Although it is possible that clone  $\lambda$ 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  $\lambda$ ATMa17.5 (S gene) and  $\lambda$ ATMa14.4 (the probable M gene) from the library of Lawn et al. (29), a cloned  $\alpha 1$ AT gene ( $\lambda$ ATM<sub>2</sub>) from an individual with the PiM<sub>2</sub>M<sub>2</sub> phenotype was examined using the four oligonucleotide probes. Analysis of the autoradiogram patterns showed, as expected, that it was identical to that of  $\lambda$ 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  $\alpha 1$ AT genes cloned from a genomic library known to contain the S type  $\alpha 1$ AT gene. Shown are two different clones (designated  $\lambda$ ATMa17.5 and  $\lambda$ ATMa14.4) of the  $\alpha 1$ AT gene isolated from the Lawn et al. (29) human genomic library. Both clones contain exons II through V of the  $\alpha 1$ AT 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<sub>M</sub>, exon III<sub>S</sub>, exon V<sub>M</sub>, and exon V<sub>Z</sub> were used for hybridization. After being washed, the nitrocellulose strips were autoradiographed for 3 h at  $-70^{\circ}\text{C}$ . As a control, on the left is shown an autoradiogram of human genomic DNA (5  $\mu\text{g}$ ) di-

gested with BglI and EcoRI and hybridized with a  $^{32}$ P-labeled  $\alpha 1$ AT 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  $\lambda$ ATMa17.5 has an apparent genotype S and cloned DNA  $\lambda$ 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  $\alpha 1$ AT 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  $\alpha 1$ AT gene using only 5  $\mu\text{g}$  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<sub>S</sub> probe, exon III<sub>M</sub> probe, exon V<sub>Z</sub> probe, and exon V<sub>M</sub> probe, the  $\alpha 1$ AT 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 M<sub>1</sub>M<sub>1</sub> yielded positive signals with exon III<sub>M</sub> and exon V<sub>M</sub> probes but negative signals with the exon III<sub>S</sub> and exon V<sub>Z</sub> probes. (b) The phenotype SS yielded positive signals with exon III<sub>S</sub> and exon V<sub>M</sub> probes but negative signals with the exon III<sub>M</sub> and exon V<sub>Z</sub> probes. (c) The phenotype ZZ yielded positive signals with exon III<sub>M</sub> and exon V<sub>Z</sub> probes but negative signals with the exon III<sub>S</sub> and exon V<sub>M</sub> probes. (d) The phenotype M<sub>1</sub>S yielded positive signals with exon III<sub>M</sub>, exon III<sub>S</sub>, and exon V<sub>M</sub> probes but a negative signal with exon V<sub>Z</sub> probe. (e) The phenotype M<sub>1</sub>Z yielded positive signals with exon III<sub>M</sub>, exon V<sub>M</sub>, and exon V<sub>Z</sub> probes but a negative signal with the exon III<sub>S</sub> probe. (f) The phenotype SZ

yielded positive signals with all four probes, i.e., the exon III<sub>M</sub>, exon III<sub>S</sub>, exon V<sub>M</sub>, and exon V<sub>Z</sub> 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<sub>M</sub> and exon III<sub>S</sub> 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<sub>M</sub> and exon V<sub>Z</sub> 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<sub>M</sub> and exon V<sub>M</sub> probes and negative signals with the exon III<sub>S</sub> and exon V<sub>Z</sub> 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, M<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub>, hybridized equally with the exon III<sub>M</sub> probe and exon V<sub>M</sub> probe, suggesting that 19-base sequences evaluated with the exon III<sub>M</sub> and exon V<sub>M</sub> probes are identical in the M<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub> 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-

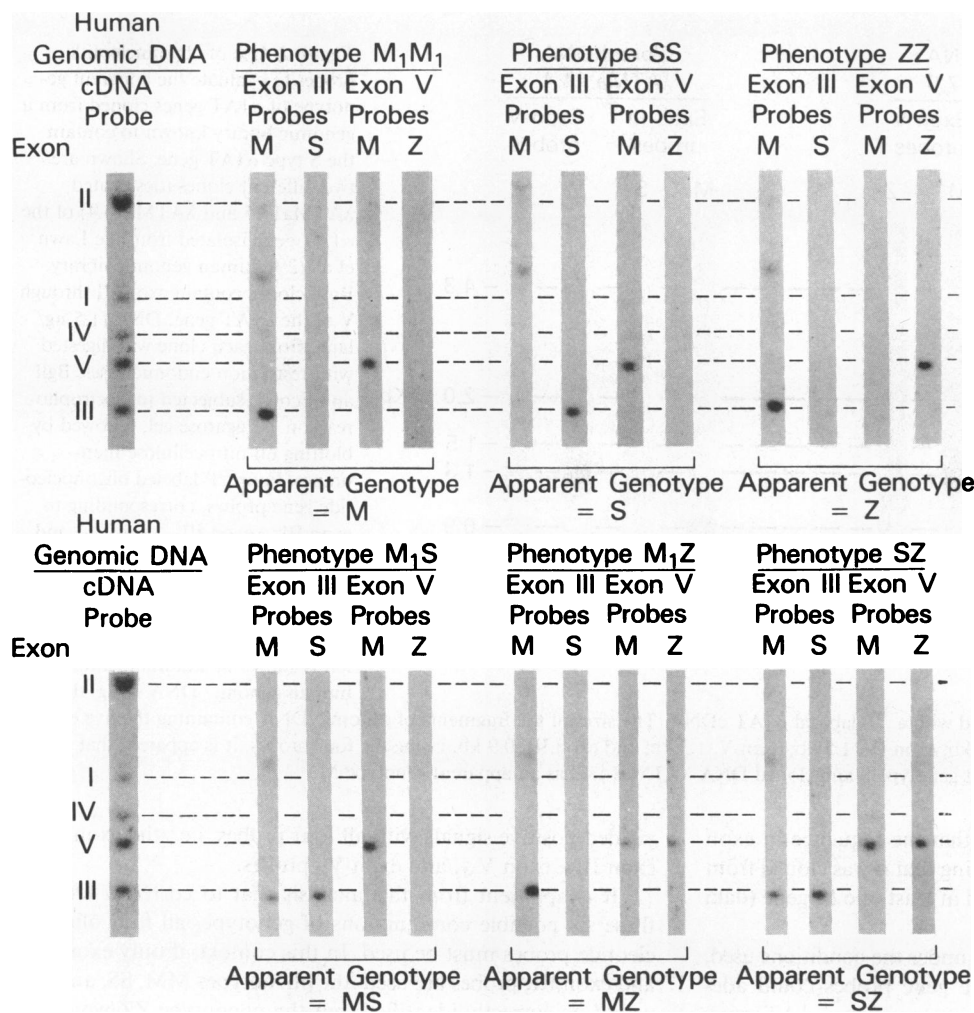


Figure 3. Use of oligonucleotide probes to correctly identify all possible combinations of M, S, and Z haplotypes. Genomic DNA (5  $\mu$ g/lane) from six different individuals, representing known phenotypes M<sub>1</sub>M<sub>1</sub>, SS, ZZ, M<sub>1</sub>S, M<sub>1</sub>Z, and SZ, were digested with the endonucleases BglI and EcoRI, electrophoresed on 1% agarose gel, blotted to nitrocellulose filters, and hybridized with <sup>32</sup>P-labeled exon III<sub>M</sub>, exon III<sub>S</sub>, exon V<sub>M</sub>, and exon V<sub>Z</sub> 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 <sup>32</sup>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  $\alpha$ 1AT types at the genomic DNA level.

Possible errors in genotypic diagnosis of the S allele using

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

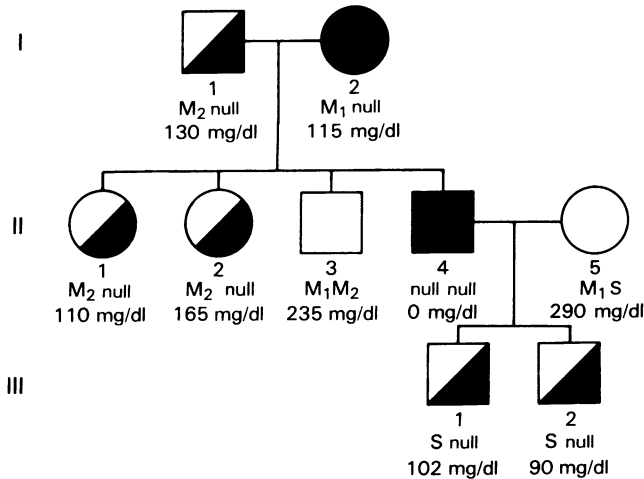
Serum phenotype*	n	Number with positive signals for each oligonucleotide probe				Correlation of serum phenotype with oligonucleotide genotype	
		Exon III probes		Exon V probes			
		M	S	M	Z	n	Percent
MM‡	11	11	0	11	0	11/11	100
SS	1	0	1	1	0	1/1	100
ZZ	12	12	0	0	12	12/12	100
MS‡	6	6	6	6	0	6/6	100
MZ‡	8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

\* Based on isoelectrofocusing,  $\alpha$ 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  $\alpha$ 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  $\alpha$ 1AT gene that does not code for a detectable  $\alpha$ 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  $\alpha$ 1AT in the serum. This individual mated with an individual with the phenotype Pi M<sub>1</sub>S and their progeny (III<sub>1</sub>, III<sub>2</sub>) both had the phenotypes Pi S null.

When the entire family was evaluated using the exon III<sub>M</sub>, exon III<sub>S</sub>, exon V<sub>M</sub>, and exon V<sub>Z</sub> 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 II<sub>4</sub>) was misidentified as "M" and the Pi M null phenotypes (I<sub>1</sub>, I<sub>2</sub>, II<sub>1</sub>, and II<sub>2</sub>) were also misidentified as "M." In contrast, the four oligonucleotide probes correctly identified the more common phenotypes MM (II<sub>3</sub>) and MS (II<sub>5</sub>). 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  $\alpha$ 1AT 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  $\alpha$ 1AT phenotype and  $\alpha$ 1AT 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  $\alpha$ 1AT 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  $\alpha$ 1AT 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  $\alpha$ 1AT 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"  $\alpha$ 1AT genotype combinations SZ and ZZ in genomic DNA. However, in the circumstance where a very rare "at risk"  $\alpha$ 1AT 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  $\alpha$ 1AT gene covered by the exon III and exon V probes, it is possible to misdiagnose an "at risk"  $\alpha$ 1AT 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  $\alpha$ 1AT genes, it is reasonable to suggest that using these four oligonucleotide probes, the vast majority of "at risk"  $\alpha$ 1AT genotype combinations can be accurately identified in genomic DNA samples, such as those obtained in prenatal studies.

**Importance of the S type  $\alpha$ 1AT gene.** The  $\alpha$ 1AT 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  $\alpha$ 1AT gene is expressed by two classes of cells, hepatocytes and mononuclear phagocytes, with hepatocytes responsible for producing most of the  $\alpha$ 1AT (35–38).

The S mutant is among the more than 30  $\alpha$ 1AT 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  $\alpha$ 1AT haplotypes are those of the M family (M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>), together representing at least 90% of all  $\alpha$ 1AT 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  $\alpha$ 1AT 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  $\alpha$ 1AT 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  $\alpha$ 1AT 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),

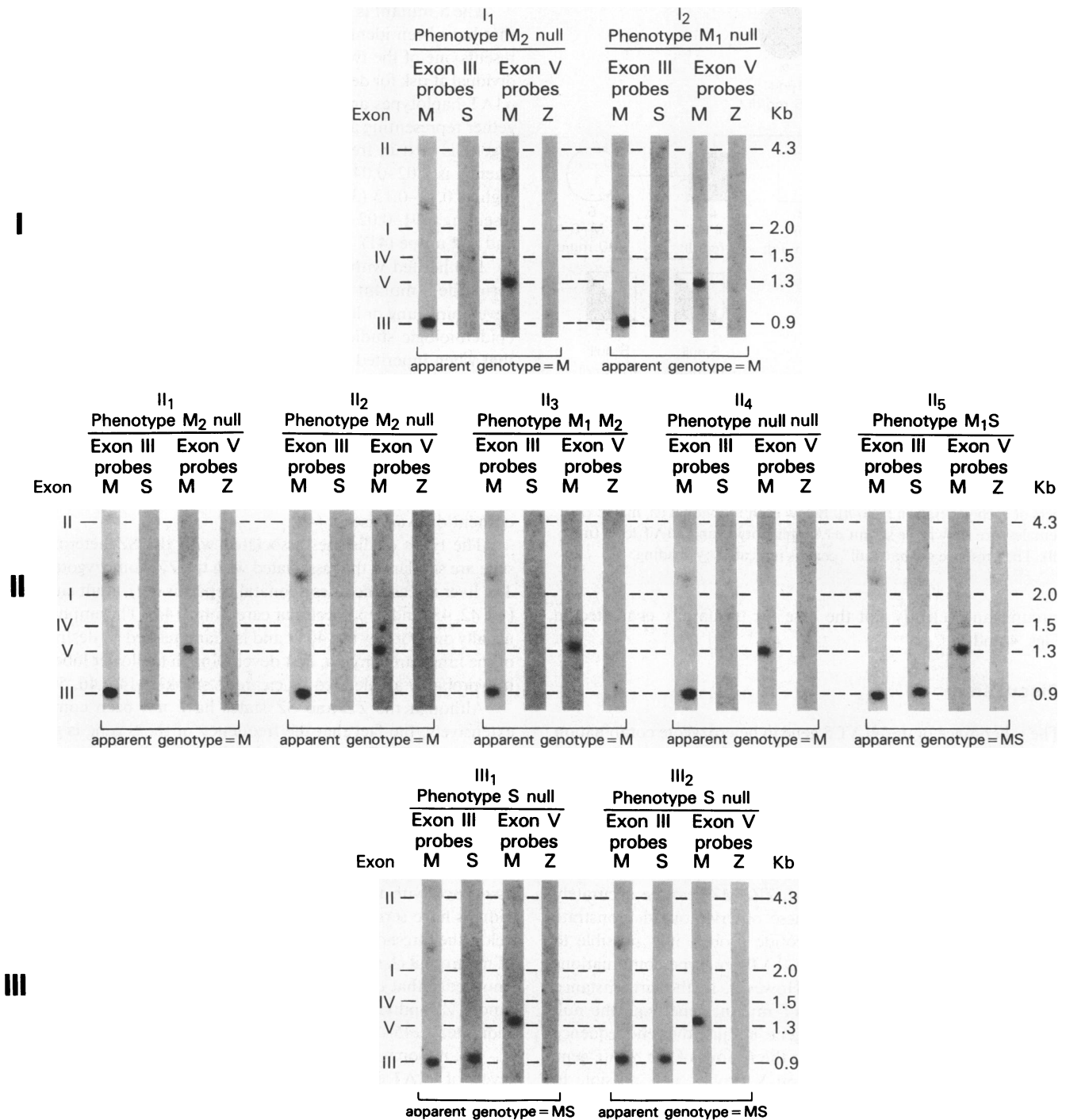


Figure 5. Evaluation of a family with the  $\alpha$ 1-antitrypsin null allele using oligonucleotide probes designed to detect exon III M and S gene sequences and exon V, M, and Z gene sequences. Total genomic DNA (5  $\mu$ g/lane) of each family member was digested with the endonucleases BglI and EcoRI, electrophoresed on 1% agarose gel, and blotted to nitrocellulose. The blotted DNA was then hybridized with  $^{32}$ P-labeled exon III<sub>M</sub>, exon III<sub>S</sub>, exon V<sub>M</sub>, and exon V<sub>Z</sub> probes, washed, and autoradiogrammed (2 d,  $-70^{\circ}\text{C}$ ). 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  $\alpha$ 1AT gene (22, 23), the  $\beta$ -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 ( $M_{\text{Pittsburg}}$ , met<sup>358</sup> to arg<sup>358</sup>) (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 I<sub>1</sub>, I<sub>2</sub>, II<sub>1</sub>, II<sub>2</sub>) 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 (II<sub>4</sub>) 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 (III<sub>1</sub>, III<sub>2</sub>) 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<sup>P</sup> (64), Pi<sup>M</sup>Duarte (65), and Pi<sup>M</sup>Malton (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.

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