α1-Antitrypsin Deficiency Caused by the α1-Antitrypsin Null$_{Mattawa}$ Gene

An Insertion Mutation Rendering the α1-Antitrypsin Gene Incapable of Producing α1-Antitrypsin

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Abstract

α1-Antitrypsin (α1AT) deficiency is a hereditary disorder associated with reduced serum α1AT levels and the development of pulmonary emphysema. An α1AT gene is defined as "Null" when no α1AT in serum is attributed to that α1AT gene. Although all α1AT Null genes have identical phenotypic consequences (i.e. no detectable α1AT in the serum), different genotypic mechanisms can cause the Null state. This study defines the molecular basis for the α1AT gene Null$_{mattawa}$, identified and cloned from genomic DNA of an individual with the Null-Null phenotype and emphysema resulting from the heterozygous inheritance of the Null$_{mattawa}$ and Null$_{bellingham}$ genes. Sequencing of exons Ic–V and all exon-intron junctions of the Null$_{mattawa}$ gene demonstrated it was identical to the common normal M1(Val^{213}) α1AT gene except for the insertion of a single nucleotide within the coding region of exon V, causing a 3′ frameshift with generation of a premature stop signal. Family analysis using oligonucleotide probes specific for the Null$_{mattawa}$ sequence demonstrated the gene was inherited in an autosomal fashion. Examination of blood monocytes demonstrated that a normal-sized, 1.8-kb α1AT mRNA transcript is associated with the Null$_{mattawa}$ gene and in vitro translation of mRNA with the Null$_{mattawa}$ mutation showed it translated at a normal rate but produced a truncated α1AT protein. Additionally, retroviral transfer of the α1AT Null$_{mattawa}$ cDNA to murine fibroblasts demonstrated no detectable intracellular or secreted α1AT, despite the presence of α1AT Null$_{mattawa}$ mRNA transcripts. These findings are consistent with the concept that the molecular pathophysiology of Null$_{mattawa}$ is likely manifested at a posttranslational level. The identification of the Null$_{mattawa}$ gene supports the concept that Null α1AT alleles represent a heterogenous group in which very different mechanisms cause the identical phenotypic state.

Introduction

α1-antitrypsin (α1AT)[1] deficiency is an autosomal hereditary disorder, characterized in adults by reduced serum α1AT levels and the development of pulmonary emphysema by ages 30–45 (1, 2). With the knowledge that the major physiologic role of α1AT is to inhibit neutrophil elastase, we understand that the pathogenesis of the emphysema associated with α1AT deficiency results from insufficient α1AT, which provides the lower respiratory tract protection against the chronic burden of elastase in the local milieu (3, 4). As a result, over several decades, there is progressive destruction of the alveolar walls, culminating in the clinical disorder, emphysema.

α1AT is coded for by a single copy gene of 12.2 kb on chromosome 14 at q31–32.3 (5–7). The coding exons of the gene are highly pleomorphic, with > 75 alleles described (1, 8, 9). The α1AT phenotype is determined by codominant expression of the parental alleles (1, 8, 10). The α1AT deficiency state associated with emphysema occurs only when there are abnormalities in both of the parental genes (8, 10, 11). In most cases there is some α1AT detectable in serum, albeit in reduced amounts (1, 2, 8, 11). For example, the commonest form of α1AT deficiency associated with emphysema results from the homozygous inheritance of the Z allele, a circumstance that results in α1AT serum levels 10–15% of normal (1, 8, 11). There are also α1AT genes causing an absolute deficiency state (10, 12–23). This category of α1AT genes, referred to as the Null alleles, represents α1AT genes in which the gene is present but does not code for an α1AT molecule that is detectable in the serum (10, 19). Compared with the deficiency alleles, the Null genes are rare, with an estimated allelic frequency of < 0.001 in Caucasians of Northern European descent (13). Although all Null alleles were originally thought to be similar, it is now recognized that the α1AT gene can be rendered Null by different mutations (21–23). The purpose of this study is to demonstrate a newly recognized type of mutational event that can cause the α1AT Null state, Null$_{mattawa}$, a nucleotide insertion in a coding exon, causing a 3′ frameshift with generation of a stop signal many codons distal to the mutation. Interestingly, unlike Null$_{bellingham}$, in which a stop codon is associated with a lack of detectable α1AT mRNA in α1AT-synthesizing cells, the Null$_{mattawa}$ gene codes for detectable α1AT mRNA, but no detectable α1AT protein produced by these cells.

Methods

Identification of the Null-Null phenotype in two index cases. The Null-Null α1AT phenotype was identified in two sisters (index case 1 and index case 2) using a combination of isoelectric focusing of serum at pH 4–5 to identify α1AT alleles, serum α1AT levels (radial immunodiffusion), and family analysis (Fig. 1) (10). To confirm that the index cases were truly Null-Null, the serum was also evaluated for the presence of α1AT using an enzyme-linked immunoassay sensitive to 2 nM[2] (24). The two index cases both had clinical evidence of emphysema.

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1. *Abbreviations used in this paper:* α1AT, α1-antitrypsin; DLCO, diffusing capacity; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; TLC, total lung capacity.

2. α1AT levels expressed in milligrams per deciliter are based on the commonly used commercial standard, whereas those in micromolar are based on a true laboratory standard; the commercial standard overestimates α1AT levels by 35% (see reference 24 for details).

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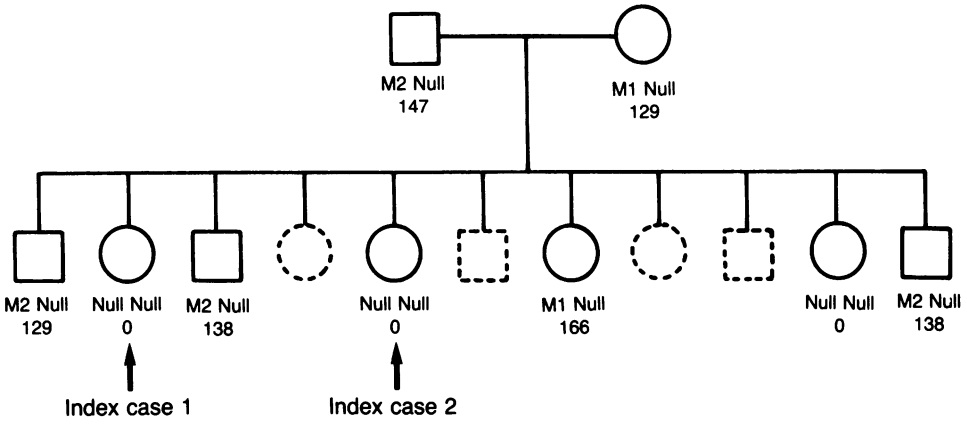

Figure 1. Family tree demonstrating the inheritance of Null alleles. α1AT phenotypes were determined by evaluating the α1AT patterns in isoelectric focusing of serum, α1AT levels in serum, and family analysis. Family members not available for evaluation are indicated by broken-lined circles or squares. The two index cases are indicated by arrows. The phenotype is listed below each family member together with the serum α1AT level (milligrams per deciliter).

Index case 1, a 39-yr-old female who had had dyspnea for 10 yr, had no smoking history. Severe pulmonary emphysema was documented by physical examination showing a hyperresonant chest and distant breath sounds; chest x ray demonstrating hyperinflation, flattened hemidiaphragms, and a marked loss of vascularity at both bases; xenon-127 ventilation scan showing abnormal retention of gas in the lower lobes and a technetium-99m perfusion scan depicting loss of vascularity in the same regions; pulmonary function testing (25) revealing that vital capacity (VC) was 42% predicted, total lung capacity (body plethysmography) was 122% predicted, forced expiratory volume in 1 s (FEV_1) was 25% predicted, FEV_1/forced (F) VC was 50% observed (69% predicted value), and diffusing capacity (DLCO; corrected for volume and hemoglobin) was 41% predicted.

Index case 2, a 31-yr-old female without pulmonary symptoms, had no smoking history. The physical examination and chest x ray were normal. The presence of mild emphysema was documented by a xenon-127 ventilation scan demonstrating abnormal retention of gas in the lower lobes, a technetium-99m perfusion scan showing complementary loss of vascularity, and lung function testing revealing VC was 93% predicted, TLC was 114% predicted, FEV_1 was 78% predicted, FEV_1/FVC was 65% observed (82% predicted), and DLCO was 87% predicted.

Evaluation of genomic DNA using oligonucleotide gene probes. To determine if the Null-Null phenotypes of the index cases were due to the inheritance of the α1AT Null alleles $Null_{bellingham}$ (21) or $Null_{granite\ falls}$ (22), genomic DNA of the index cases and relevant controls were evaluated using gel hybridization with oligonucleotide gene probes by the method of Kidd et al. (26) as modified by Satoh et al. (21). Genomic DNA of index case 1, a normal M1(Val^{213}) homozygote (27), a $Null_{bellingham}$ homozygote, and an M1(Val^{213}) $Null_{granite\ falls}$ heterozygote were digested with the restriction endonuclease Pst I and the DNA was electrophoresed on 0.7% agarose (8 μg/lane). The gels were hybridized at 55°C with oligonucleotide gene probes centered on the sequence of interest (see Fig. 2, *top* for the sequences used). The oligonucleotide probes were 5′-labeled with [^{32}P]ATP and separated from unincorporated label on an 8% polyacrylamide-8 M urea denaturing gel. After hybridization, the gels were washed at 60°C in 25 mM sodium phosphate buffer, pH 7.0, 0.45 M NaCl, 2.5 mM EDTA, 0.1% SDS for 15 min and autoradiographed at −70°C for 2 wk.

Cloning and sequencing of the $Null_{mattawa}$ gene. After oligonucleotide analysis of genomic DNA of index case 1 demonstrated that the Null-Null state in this family was caused by inheritance of the $Null_{bellingham}$ allele together with a previously unidentified Null allele (referred to subsequently as $Null_{mattawa}$ based on the birthplace of index case 1). The $Null_{mattawa}$ gene was cloned from genomic DNA of index case 1 by conventional methods. Using DNA from skin fibroblasts, a cosmid library was prepared by inserting size-fractionated partial Mbo I-digested DNA into the vector C2RB (28) with subsequent packaging and transfection into *Escherichia coli* 1046 as previously described by van Ommen et al. (29). The primary library was plated out on 20 90-mm dishes at a density of 40,000 colonies per dish. Duplicate filters were prepared and the filters hybridized under standard conditions using a ^{32}P-labeled human α1AT cDNA probe. Clones containing the region of the α1AT gene were confirmed by restriction enzyme analysis using Eco RI. To select for the $Null_{mattawa}$ allele, cosmid clones containing the α1AT gene were evaluated using oligonucleotide gene probes as described above. Because the genotype of index case 1 had been defined as heterozygous $Null_{bellingham}$ $Null_{mattawa}$, those clones which did not contain the $Null_{bellingham}$ sequence at residue 217 by definition contained the $Null_{mattawa}$ allele. A single 40-kb cosmid clone that contained the entire α1AT $Null_{mattawa}$ gene plus flanking regions was selected and subcloned into pUC19 by standard techniques (30). The recombinant subclones obtained contained 0.5 kb encompassing exon Ic, 1.6 kb encompassing exon II, 2.4 kb encompassing exons III and IV, and 1.1 kb encompassing exon V (see Fig. 2, *top*, for the overall normal α1AT gene structure). These subclones were used as templates for sequencing using the dideoxynucleotide chain termination method (31) with bidirectional primers of 15-mer oligonucleotides (30). Sequencing included 150 bp 5′ to exon Ic, exons Ic–V together with the intron-exon junctions, and 40 bp 3′ to exon V.

Demonstration of inheritance of the $Null_{mattawa}$ allele. To demonstrate the inheritance of the $Null_{mattawa}$ mutation, oligonucleotide probes were prepared that were complementary to both the region centered at residue 353 in exon V with the insertional mutation in $Null_{mattawa}$ and to the normal sequence in this region. Genomic DNA from index case 1, index case 2, the father of the index cases, and an M1(Val^{213}) homozygote control were cut with Pst I and evaluated with labeled oligonucleotide probes using the methods described above.

Evaluation of α1AT mRNA transcripts from cells expressing the α1AT gene. To gain insight into the consequences of the $Null_{mattawa}$ mutation, blood monocytes, cells known to normally express the α1AT gene (32, 33) were evaluated for the presence of α1AT mRNA transcripts. Monocytes were evaluated from index case 2 and compared with those from a normal M1(Val^{213}) homozygote control. Although index case 2 (like index case 1) is a $Null_{bellingham}$-$Null_{mattawa}$ heterozygote, the consequences of the $Null_{mattawa}$ allele could be directly evaluated independent of the $Null_{bellingham}$ allele because (*a*) the parental α1AT genes are codominantly expressed, i.e., α1AT gene expression of one allele is independent of the other parental allele (1, 8, 10); and (*b*) the $Null_{bellingham}$ gene is known to be associated with no α1AT mRNA transcripts and no production of α1AT by blood monocytes (20).

Blood monocytes were isolated from index case 2 and a normal M1(Val^{213}) homozygote using adherence purification of mononuclear cells obtained by monocytapheresis. Blood mononuclear leukocytes were harvested by cytapheresis on a cell separator (model 2297; IBM Instruments, Inc., Danbury, CT) during continuous centrifugation by standard techniques (34). The mononuclear cells were separated from contaminating granulocytes using Hypaque-Ficoll density centrifugation. The resulting mononuclear cells were resuspended in DMEM supplemented with 10% FCS (Biofluids) and allowed to adhere to

150-mm tissue culture plates for 1 h at 37°C. The nonadherent cells were removed and the plates were washed three times with PBS, pH 7.4. The resulting adherent cells were harvested by scraping, washed three times, with PBS, and replated as above. The resulting cell populations were > 90% monocytes (morphology and nonspecific esterase staining) and had > 95% viability (trypan blue exclusion). α1AT mRNA transcripts in the monocytes were evaluated by Northern analysis (33). Total cellular RNA (10 μg/lane) prepared by guanidine HCl extraction followed by CsCl centrifugation was electrophoresed in agarose gels under denaturing conditions, transferred to nitrocellulose filters, hybridized with a ^{32}P-labeled α1AT cDNA probe, and autoradiographed.

Analysis of translation of Null$_{mattawa}$ α1AT mRNA transcripts. In vitro translation analysis was used to examine the effect of the Null$_{mattawa}$ mutation on the translation of the α1AT mRNA transcript into protein. To accomplish this, an α1AT Null$_{mattawa}$ cDNA was constructed from a normal α1AT M1(Val^{213}) cDNA (pPB01) by standard techniques. Both this mutant α1AT cDNA and a normal M1(Val^{213}) α1AT cDNA were used to construct plasmids for use in the Riboprobe (Promega-Biotec) SP6 polymerase in vitro transcription system. The M1(Val^{213}) α1AT cDNA containing plasmid and the Null$_{mattawa}$ α1AT cDNA containing plasmid served as templates for generation of capped, poly-adenylated synthetic α1AT mRNA transcripts of normal M1(Val^{213}) type or mutant Null$_{mattawa}$ type, respectively (35). After purification, 1 μg of each α1AT mRNA species was used to direct translation (1 h at 37°C) in a rabbit reticulocyte lysate system (Promega-Biotec) with [^{35}S]methionine used as a label. Aliquots of the reaction mixtures were analyzed by SDS-acrylamide gel electrophoresis and fluorography and quantified by laser densitometry.

Retroviral gene transfer of human M1(Val^{213})-type and Null$_{mattawa}$-type α1AT cDNAs. To examine posttranslational events in α1AT biosynthesis, M1(Val^{213}) and Null$_{mattawa}$ type α1AT cDNAs were transferred to murine fibroblasts using retroviral gene transfer. The retroviral vectors used to insert the full length M1(Val^{213})-type and Null$_{mattawa}$-type α1AT cDNAs into the genome of mouse fibroblasts were constructed from the N2 vector as previously described (36). The final retroviral vectors (pN2-α1AT/M1 and pN2-α1AT/Mattawa) contained (5′ to 3′) the SV40 early promoter and full length human α1AT cDNAs of M1(Val^{213}) type or Null$_{mattawa}$ type, respectively, inserted into the Xho I site of N2. Sequence analysis confirmed that pN2-α1AT/Mattawa differed from pN2-α1AT/M1 by only the single nucleotide insertion corresponding to the Null$_{mattawa}$ mutation. The helper virus-free packaging cell line ψ_2 (37) was used to package transcripts from pN2-α1AT/M1 and pN2-α1AT/Mattawa into ecotropic infectious viral particles that were then used to infect NIH-3T3 cells. Polyclonal populations of infected NIH-3T3 cells were scored at > 100 colonies/10-cm plate and were equivalent for the human α1AT M1(Val^{213})-type and Null$_{mattawa}$-type cDNA containing cell populations, referred to as NIH-3T3/α1AT-M1 and NIH-3T3/α1AT-Mattawa, respectively.

α1AT mRNA levels in mouse fibroblasts. Human α1AT mRNA transcripts were identified in NIH-3T3/α1AT-M1 and NIH-3T3/α1AT-Mattawa cells by cytoplasmic dot hybridization (38). Uninfected NIH-3T3 cells were used as control. Extracted cytoplasmic RNA was denatured and applied to nitrocellulose filters with a minifold apparatus (Schleicher & Schuell, Keene, NH) in serial dilutions. The filters were hybridized using a ^{32}P-labeled human α1AT cDNA probe. Exposure of the autoradiograms was for 48 h at −70°C.

Synthesis and secretion of human α1AT by mouse fibroblasts. The synthesis and secretion of human α1AT by NIH-3T3/α1AT-M1 and NIH-3T3/α1AT-Mattawa cells was evaluated by immunoprecipitation of [^{35}S]methionine-labeled α1AT as previously described (36). NIH-3T3/α1AT-M1 and NIH-3T3/α1AT-Mattawa cells were plated (5×10^5 cells per 60-mm plate); the next day, each plate was incubated for 10 min in methionine-free Iscove's minimal essential medium containing 10% dialyzed calf serum, pulsed with 250 μCi of [^{35}S]-methionine (600 Ci/mmol) for 30 min, then chased with nonselective media supplemented with 1 mM unlabeled methionine for 120 min. To normalize the two study cell populations, aliquots of supernatants containing 10^5 dpm and lysates containing 10^6 dpm of TCA-precipitable protein were compared. Immunoprecipitation, gel electrophoresis, and fluorography were as previously described (33).

Results

Evaluation of index case 1 for the presence of the Null$_{granite\ falls}$ and the Null$_{bellingham}$ alleles. Using oligonucleotide probes specific for the sequence defining the Null$_{granite\ falls}$ mutation, the Null$_{bellingham}$ mutation, and the corresponding normal sequences of these regions, it was apparent that the α1AT genotype of index case 1 was heterozygous Null-Null containing the Null$_{bellingham}$ mutation at one allele and a different α1AT Null mutation in the other allele (Fig. 2). In this regard, genomic DNA of index case 1 had a sequence that was normal in the regions defining the Null$_{granite\ falls}$ mutation (compare lane *6* with lane *5*, along with the controls in lanes *1–4*). In contrast, the genomic DNA of index case 1 clearly contained the Null$_{bellingham}$ mutation in one gene (lane *12*), and a normal sequence in the Null$_{bellingham}$ mutation region in the other (lane *11*, along with controls lanes *7–10*). Together, these findings indicate that index case 1 must be a heterozygous Null-Null, with one Null$_{bellingham}$ allele and another, different α1AT Null allele, which possesses the normal sequence at positions 214–220 (the region of the Null$_{bellingham}$ mutation).

Identification of the Null$_{mattawa}$ mutation by sequence analysis. Sequencing of exons Ic–V, all exon-intron junctions, and the 3′ flanking region of the cloned α1AT Null$_{mattawa}$ gene of index case 1 demonstrated that it was identical to that of the normal M1(Val^{213}) α1AT gene except for a single nucleotide insertional mutation in the exon V coding region at residue 353 of the primary amino acid sequence (Fig. 3). The 217 region of the Null$_{mattawa}$ allele, the region of the Null$_{bellingham}$ mutation site, was similar to the normal α1AT M1(Val^{213}) gene. This novel mutation causes a 3′ frameshift, resulting in an altered reading frame commencing at the codon for amino acid 353. The new nucleotide sequence codes for a significantly altered amino acid sequence distal to the insertion site and terminates in a premature stop signal at new codon 376.

Demonstration of inheritance of the Null$_{mattawa}$ gene. Evaluation of genomic DNA of the index cases and the father of the index cases using synthetic oligonucleotide probes centered at the 353 region and specific for the Null$_{mattawa}$ mutation sequence or the corresponding normal sequence, demonstrated that the Null$_{mattawa}$ nucleotide insertion mutation had been inherited in an autosomal fashion (Fig. 4). In this context, specific hybridization of the Null$_{mattawa}$ probe to the genomic DNA of index case 1 (lane *2*), index case 2 (lane *4*), and the father (lane *6*), yielded a 1.1-kb band corresponding to the Pst I fragment containing the mutated sequence in exon V. Genomic DNA of both index cases and the father also specifically hybridized with the normal 350–356 probe. In the case of the father, the alternate allele is M2 (Fig. 1) and thus is identical to the normal probe in the 350–356 region. Likewise, even though both index cases have the Null$_{bellingham}$ allele as the alternate allele, they are normal in the 350–356 region (the Null$_{bellingham}$ substitution mutation is centered at residue 217). Based on this analysis, it may be deduced that the genotype of the mother is M1Null$_{bellingham}$ despite the lack of direct analysis of her DNA. Together, these observations confirm both the

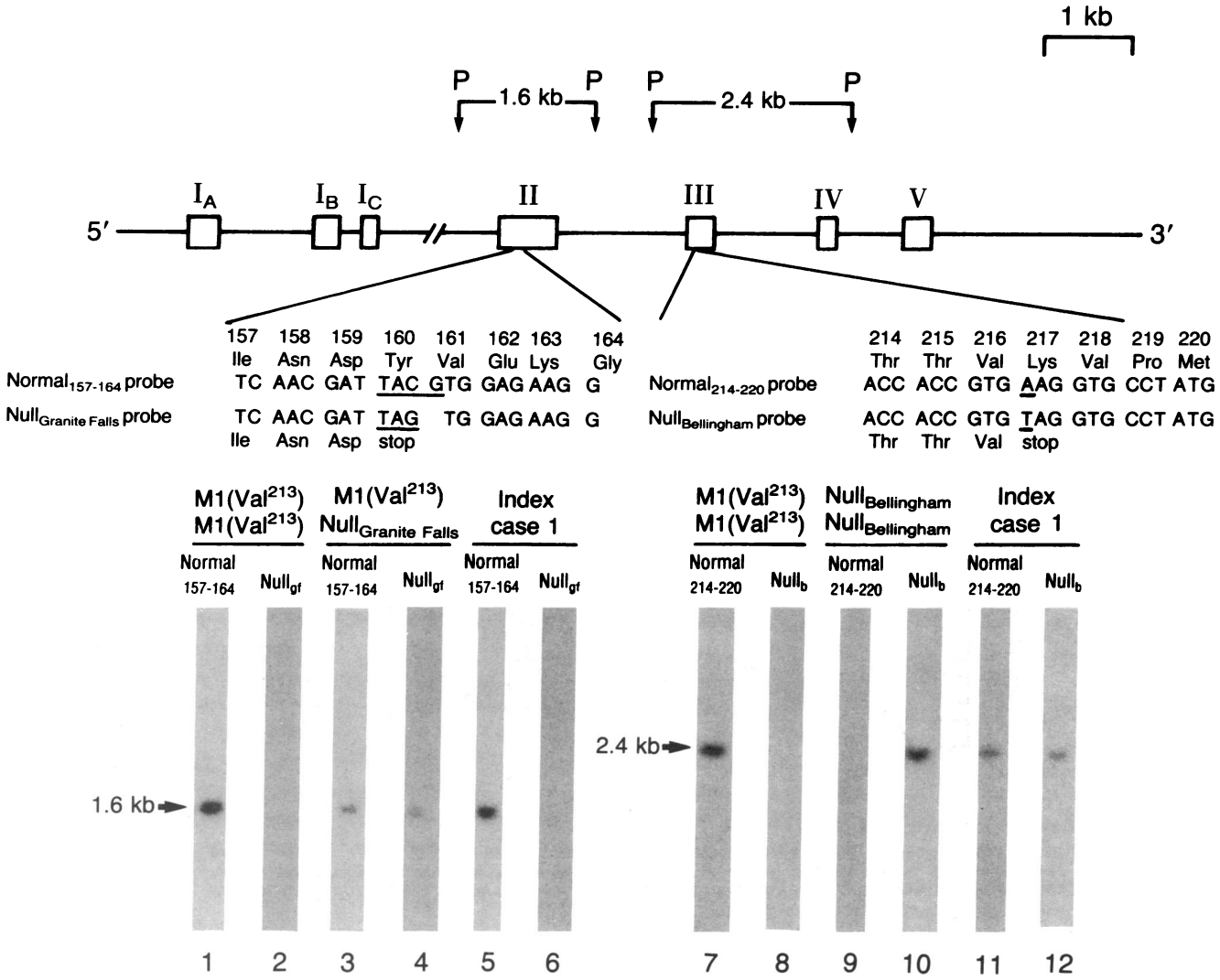

Figure 2. Evaluation of index case 1 for the presence of two previously described Null alleles, $Null_{granite\ falls}$ (22) and $Null_{bellingham}$ (21). The oligonucleotide gene probes used included: probes centered on the $Null_{granite\ falls}$ defect ($Null_{gf}$) or the corresponding normal sequence for residues 157–164 ($Normal_{157-164}$) and probes that centered on the $Null_{bellingham}$ defect ($Null_b$) or the corresponding normal sequence for residues 214–220 ($Normal_{214-220}$). Shown at the top is the structure of the α1AT gene. Exons I_A–I_C contain untranslated regions (for hepatocyte α1AT expression, the 3′ part of exon I_C and a short 5′ segment of exon II; for mononuclear phagocyte expression, I_A, I_B, I_C and a short 5′ segment of exon II; see references 6, 7, and 9), whereas the coding regions for the protein are in exons II–V (6). Both the sequences of the oligonucleotide probes and the corresponding coded amino acids in the primary protein structure are depicted; the mutational changes are underlined. The probes were labeled and used to evaluate Pst I- (P) digested genomic DNA from various sources; Pst I was used because it separates the exons with the $Null_{granite\ falls}$ and $Null_{bellingham}$ mutations (see diagram and text). Lane *1*, genomic DNA of an M1(Val^{213}) homozygote control evaluated with $Normal_{157-164}$ probe. Lane *2*, identical to lane *1* except evaluated with $Null_{granite\ falls}$ probe. Lane *3*, genomic DNA of M1(Val^{213}) $Null_{granite\ falls}$ heterozygote evaluated with $Normal_{157-164}$ probe. Lane *4*, identical to lane *3* except evaluated with $Null_{granite\ falls}$ probe. Lane *5*, genomic DNA of index case 1 (see Fig. 1) evaluated with $Normal_{157-164}$ probe. Lane *6*, identical to lane *5* except evaluated with $Null_{granite\ falls}$ probe. Lane *7*, genomic DNA of an M1(Val^{213}) homozygote control evaluated with $Normal_{214-220}$ probe. Lane *8*, identical to lane *7* except evaluated with $Null_{bellingham}$ probe. Lane *9*, genomic DNA of $Null_{bellingham}$ homozygote evaluated with $Normal_{214-220}$ probe. Lane *10*, identical to lane *9* except evaluated with $Null_{bellingham}$ probe. Lane *11*, genomic DNA of index case 1 evaluated with $Normal_{214-220}$ probe. Lane *12*, identical to lane *11* except evaluated with $Null_{bellingham}$ probe.

autosomal inheritance of the $Null_{mattawa}$ allele in this family and the heterozygous $Null_{bellingham}$-$Null_{mattawa}$ phenotype of the index cases.

Evaluation of α1AT-producing cells for mRNA transcripts associated with the $Null_{mattawa}$ gene. Because the $Null_{bellingham}$ allele is associated with the complete absence of α1AT mRNA in α1AT producing cells (20), any α1AT mRNA transcript exhibited by blood monocytes of the index case must represent the exclusive expression of the $Null_{mattawa}$ allele. In this regard, Northern analysis of RNA of index case 2 showed that α1AT mRNA transcript was expressed (Fig. 5). Furthermore, it was 1.8 kb long (lane *2*), similar to the α1AT mRNA transcripts of a normal M1(Val^{213}) homozygote (lane *1*). It appears, therefore, that the presence of a stop codon near the 3′ end of the α1AT mRNA molecule generated by the $Null_{mattawa}$ mutation is not associated with the absence of α1AT mRNA as is the case for the $Null_{bellingham}$ mutation, in which the stop signal is generated near the center of the coded α1AT mRNA.

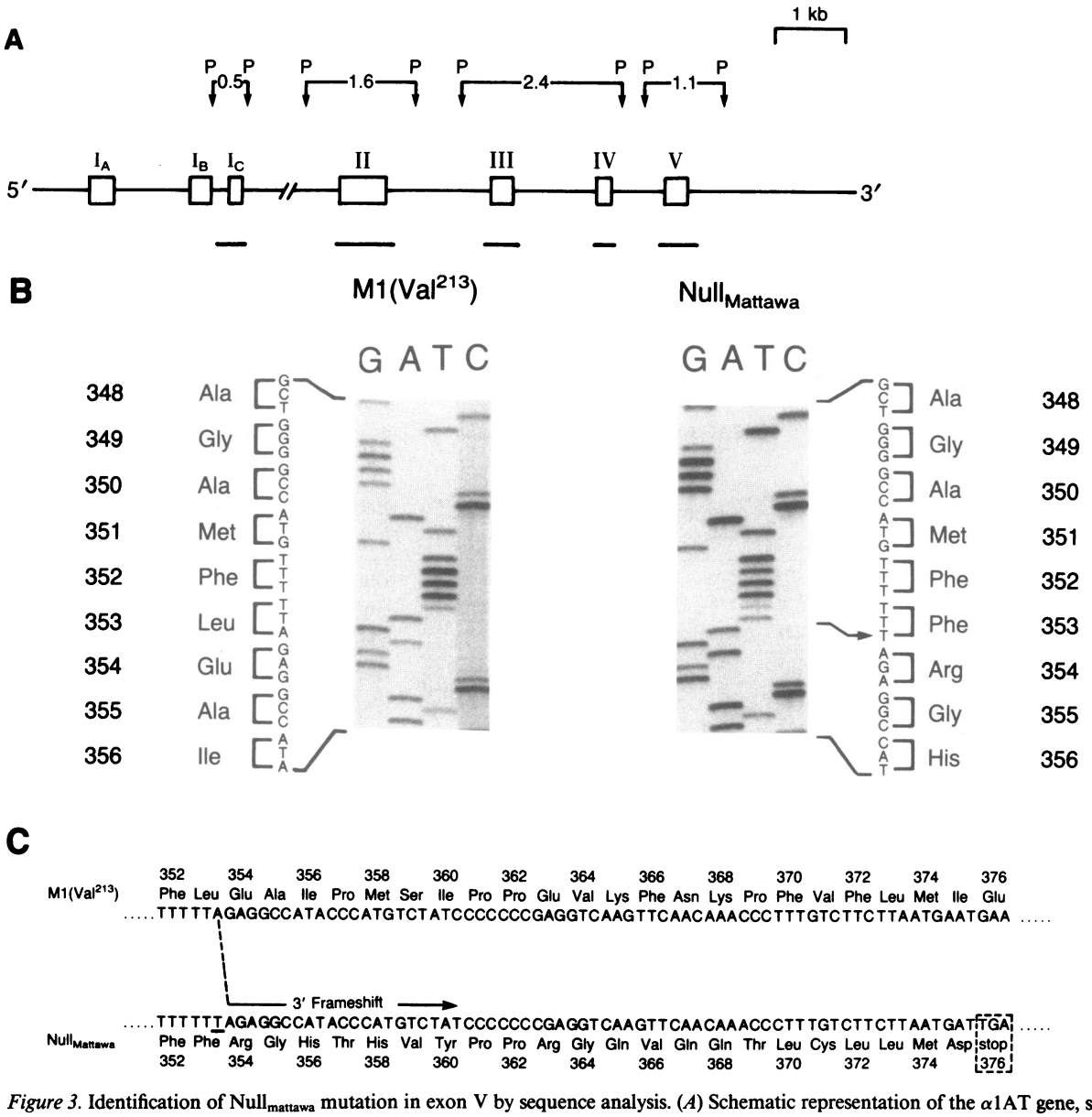

Figure 3. Identification of $Null_{mattawa}$ mutation in exon V by sequence analysis. (*A*) Schematic representation of the α1AT gene, see legend to Fig. 2 for details. The Pst I (P) subclones used for sequencing are indicated above the gene; the actual areas sequenced are shown as solid lines below the gene. The sequence was identical to that of the α1AT M1(Val^{213}) gene except for a region in exon V. (*B*) Evaluation of the 348–356 region of exon V for the M1(Val^{213}) and $Null_{mattawa}$ alleles. A thymidine insertion (*arrow*) in the codon for amino acid 353 is the single change in the $Null_{mattawa}$ allele. (*C*) The nucleotide insertion in codon 353 causes a frameshift in the 3′ direction, which alters the reading frame and generates a stop signal at the new codon 376.

Translation of the $Null_{mattawa}$ α1AT mRNA transcript. Evaluation of the translation of the $Null_{mattawa}$ α1AT mRNA transcript demonstrated that it was capable of directing the synthesis of an α1AT protein product in a fashion comparable to the normal M1(Val^{213}) α1AT mRNA transcript, except that, as expected for a mutation causing a stop codon, the translation product was truncated (Fig. 6). In this context, in vitro translation of a synthetic α1AT M1(Val^{213}) mRNA transcript directed synthesis of a 47-kD α1AT protein species (lane *1*), corresponding to the nonglycosylated α1AT protein including the 24-amino acid signal peptide. In contrast, the α1AT $Null_{mattawa}$ mRNA transcript directed the synthesis of a 45-kD α1AT protein species (lane *2*), corresponding to the primary translation product of α1AT minus the 19 amino acids 3′ to the premature termination codon of the $Null_{mattawa}$ gene that are not translated into protein. Quantification of the amounts of α1AT directed by the $Null_{mattawa}$ mRNA demonstrated it was similar to that directed by the normal mRNA ($P > 0.2$; two-tailed t test). It thus appears that the insertional mutation of the $Null_{mattawa}$ gene does not alter the capacity of the $Null_{mattawa}$ mRNA transcript to be translated into protein and that, therefore, the molecular pathophysiology accounting for the α1AT Null state associated with this gene is likely not manifest at the translational level.

Human α1AT gene expression in murine fibroblasts modified to contain the human α1AT of M1(Val^{213})-type and $Null_{mattawa}$-type cDNAs. Retroviral gene transfer of the human α1AT cDNAs of the normal M1(Val^{213})-type and the mutant

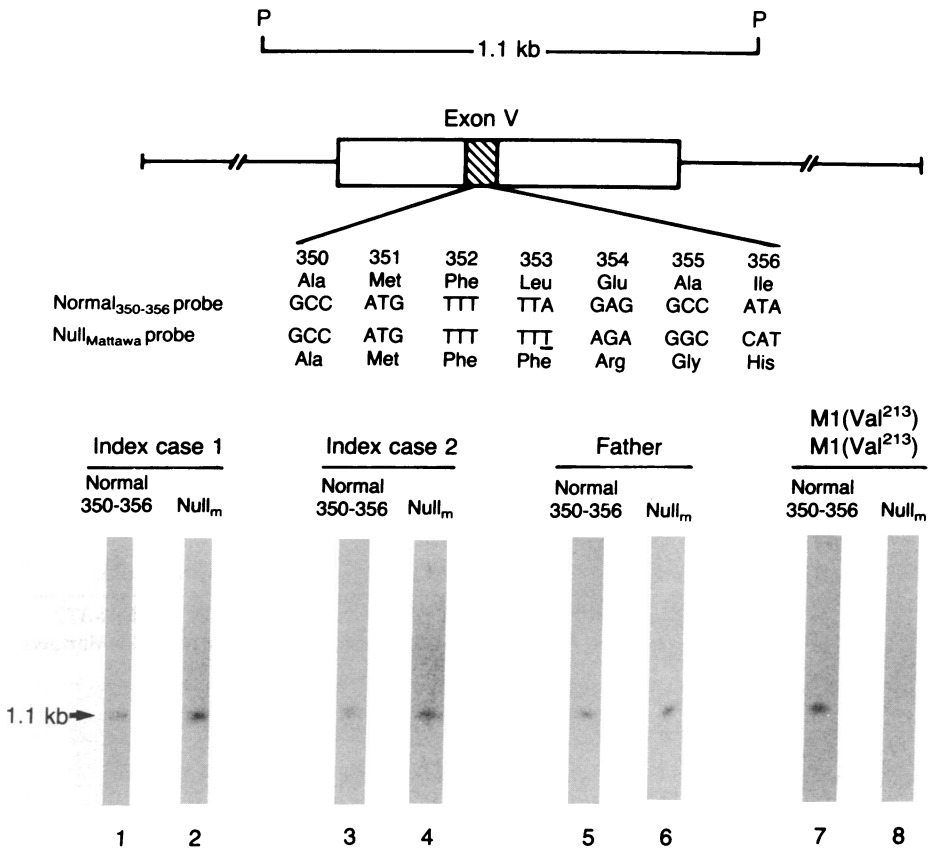

Figure 4. Determination of the inheritance of the $Null_{mattawa}$ allele using an oligonucleotide probe specific for the $Null_{mattawa}$ mutation. Synthetic 21-mer oligonucleotide probes centered in exon V about the $Null_{mattawa}$ mutation ($Null_m$) or the corresponding normal sequence in the region coding for residues 350–356 ($Normal_{350-356}$) were used to evaluate genomic DNA. Shown are the sequences of the probes and the corresponding coded amino acid sequence of the primary protein structure. The nucleotide insertion in $Null_{mattawa}$ is underlined. Genomic DNA was digested with Pst I (P), an enzyme that cuts on either side of exon V, rendering a 1.1-kb fragment. Lane *1*, genomic DNA of index case 1 evaluated with the $Normal_{350-356}$ probe. Lane *2*, identical to lane 1 except evaluated with the $Null_{mattawa}$ probe. Lane *3*, genomic DNA of index case 2 evaluated with the $Normal_{350-356}$ probe. Lane *4*, identical to lane *3* except evaluated with the $Null_{mattawa}$ probe. Lane *5*, genomic DNA of the father of the index cases evaluated with the Normal 350–356 probe. Lane *6*, identical to lane 5 except evaluated with the $Null_{mattawa}$ probe. Lane *7*, genomic DNA of M1(Val^{213}) homozygote control evaluated with the Normal 350–356 probe. Lane *8*, identical to lane *7* except evaluated with the $Null_{mattawa}$ probe.

$Null_{mattawa}$ type to murine fibroblasts demonstrated that the α1AT Null state associated with the $Null_{mattawa}$ gene could be reproduced in cells that do not normally produce human proteins, and do not normally produce any form of α1AT (Fig. 7). Cells modified to contain the normal α1AT M1(Val^{213})-type cDNA exhibited human α1AT gene expression at the mRNA and protein level. In contrast, whereas the cells modified to contain the α1AT $Null_{mattawa}$-type cDNA expressed equivalent amounts of α1AT mRNA transcripts, no α1AT protein product could be detected. In this regard, cytoplasmic dot analysis of RNA isolated from the cell populations using a ^{32}P-labeled α1AT cDNA probe demonstrated comparable levels of α1AT mRNA transcripts in the NIH-3T3/α1AT-M1 and NIH-3T3/α1AT-Mattawa cells (Fig. 8 *A*). This finding is consistent with the concept that the $Null_{mattawa}$ α1AT mRNA transcript has comparable stability to the normal M1(Val^{213}) α1AT mRNA

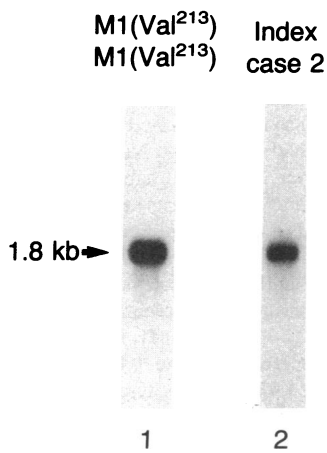

Figure 5. Northern analysis of α1AT transcripts in blood monocytes of index case 2 and M1(Val^{213}) homozygote control. Total cellular RNA (10 μg/lane) was evaluated using a ^{32}P-labeled human α1AT cDNA probe. Lane *1*, M1(Val^{213}) homozygote. Lane *2*, index case 2. The position and average length of normal M1(Val^{213}) α1AT mRNA transcript is indicated.

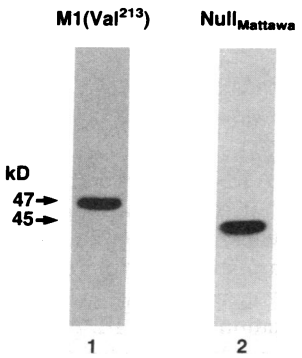

Figure 6. In vitro translation of $Null_{mattawa}$ and M1(Val^{213}) α1AT mRNA transcripts. Synthetic α1AT mRNA transcripts derived by in vitro transcription of $Null_{mattawa}$ and M1(Val^{213}) α1AT-type cDNAs, respectively, were used to direct α1AT protein synthesis in a rabbit reticulocyte lysate system with [^{35}S]methionine as a label. Shown are fluorograms of SDS-acrylamide gel analysis of [^{35}S]methionine-labeled α1AT. Lane *1*, M1(Val^{213}) α1AT. Lane *2*, $Null_{mattawa}$ α1AT. The positions of the 47- and 45-kD primary translation products of M1(Val^{213}) and $Null_{mattawa}$ mRNAs, respectively, are indicated.

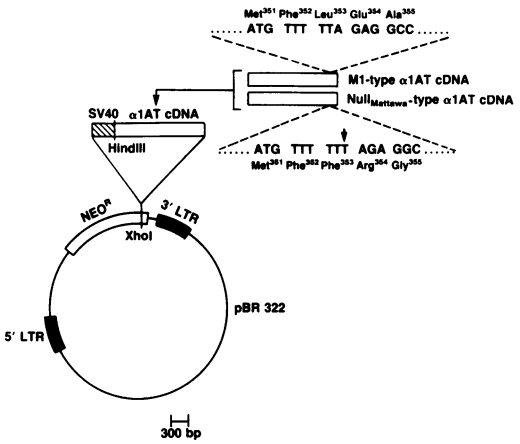

Figure 7. DNA plasmid map of the pN2-α1AT/M1 and pN2-α1AT/Mattawa retroviral vectors containing full-length human α1-antitrypsin cDNAs of M1-type and $Null_{mattawa}$-type, respectively. The pN2-α1AT/M1 and pN2-α1AT/Mattawa vectors were formed from pN2 by combining (5′–3′) the SV40 early promoter and the forward orientation of the α1AT cDNAs of M1(Val^{213}) type and $Null_{mattawa}$ type, respectively, into the Xho I site. The M1(Val^{213})-type and $Null_{mattawa}$-type α1AT cDNAs differ by a single-nucleotide insertion causing the replacement of leucine by phenylalanine at amino acid position 353 of the coded protein and a 3′ frameshift with the generation of a premature stop codon at position 376.

transcript. However, despite the presence of α1AT mRNA transcripts which differ from M1(Val^{213}) α1AT mRNA transcripts by only a single nucleotide insertion, murine fibroblasts containing the $Null_{mattawa}$-type cDNA exhibited no detectable human α1AT protein (Fig. 8 *B*). In this context, labeling with [^{35}S]methionine for 30 min followed by a 120-min chase showed that the murine fibroblasts containing the M1(Val^{213})-type α1AT cDNA secreted a 52-kD mature form of human α1AT specifically immunoprecipitated by anti–α1AT antibody (lane *1*). In marked contrast, no secreted human α1AT could be detected from the $Null_{mattawa}$ cDNA-containing cells (lane *2*). Analysis of the cellular lysates after a 30-min pulse labeling with [^{35}S]methionine for the NIH-3T3/α1AT-M1 cells showed a 50-kD precursor form of human α1AT specifically immunoprecipitated by anti–α1AT antibody (lane *3*). In contrast, examination of lysates of NIH-3T3/α1AT-Mattawa cells demonstrated no detectable intracellular α1AT (lane *4*). Thus, although it is conceivable that the truncated α1AT protein associated with the $Null_{mattawa}$ gene is stable but not detected by the polyclonal anti-α1AT antibody used, it is more likely that if a truncated protein is produced, it is degraded rapidly and thus not detected.

Discussion

Although the consequences of all α1AT Null alleles are identical, i.e., no detectable serum α1AT, it is becoming apparent that several different mutational events may render an α1AT gene Null. To date, all result from mutations in exons coding for the mature form of the α1AT protein. $Null_{bellingham}$ results from a nucleotide substitution mutation in exon III, causing the substitution of a stop signal for a lysine residue (Lys^{217} AAG → $stop^{217}$ TAG) (21). In contrast, $Null_{granite\ falls}$ results from a single nucleotide deletion mutation in exon II at amino acid position 160 causing a 5′ frameshift of the reading frame with the consequent generation of a premature stop signal at the codon position of the mutation (Tyr^{160}TAC Val^{161}GTG, deletion of C in codon TAC for residue 160, and 5′ frameshift forming $stop^{160}$TAG) (22). The $Null_{hong\ kong}$ allele is a TC dinucleotide deletion at Leu^{318} causing a 5′ frameshift with generation of a premature termination codon at position 334 (23). The α1AT $Null_{mattawa}$ allele presents yet another mechanism; a Null allele resulting from a single-nucleotide insertional mutation within the coding region of exon V, causing a distal 3′ frameshift, different amino acids substituting for residues 353 → 375, and a premature stop codon at residue 376. Thus, the heterogeneity in the Null mutations is diverse. Consistent with this concept, we have recently observed an entirely different cause of the Null state, an inherited deletion of the entire α1AT gene ($Null_{deletion\ procida}$; Takahashi, H., and R. Crystal, unpublished observation).

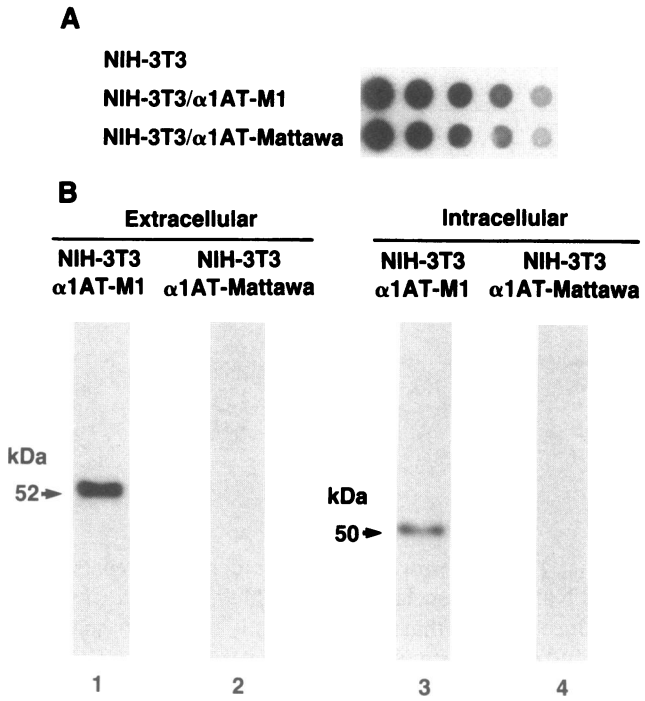

Figure 8. Evaluation of human α1AT gene expression in murine fibroblasts modified to contain the α1AT cDNAs of M1(Val^{213})-type and $Null_{mattawa}$-type. (*A*) Identification of α1AT mRNA transcripts in mouse fibroblasts containing the integrated M1(Val^{213})-type and $Null_{mattawa}$-type human α1AT cDNAs. Shown are data from NIH-3T3/α1AT-M1 and NIH-3T3/α1AT-Mattawa cells and as control, unmodified NIH-3T3 cells. Cytoplasmic RNA was evaluated for human α1AT mRNA transcripts using cytoplasmic dot hybridization analysis with a ^{32}P-labeled α1AT cDNA probe. (*B*) Synthesis and secretion of human α1AT by mouse fibroblasts modified to contain the human α1AT cDNAs of M1(Val^{213})-type and $Null_{mattawa}$-type. Shown are fluorograms of SDS-acrylamide gel analysis of ^{35}S-labeled proteins isolated from lysates and supernatants of NIH-3T3/α1AT-M1 and NIH-3T3/α1AT-Mattawa cells (30 min pulse with [^{35}S]methionine and 120 min chase with label-free media) and immunoprecipitated with an anti–α1AT antibody. Lysates were analyzed directly after the 30-min pulse period and supernatants after the 120-min chase period. Lane *1*, NIH-3T3/α1AT-M1, supernatant. Lane *2*, NIH-3T3/α1AT-Mattawa, supernatant. Lane *3*, NIH-3T3/α1AT-M1, lysate. Lane *4*, NIH-3T3/α1AT-Mattawa, lysate. The position of the 52-kD secreted form and 50-kD intracellular form of α1AT are indicated.

Insertional mutations generating a Null state have been described in other protein deficiency disorders. Subsets of the β^0 thalassemia genes have been identified in ethnic Asian Indians and Chinese that result from different single nucleotide insertional mutations within the coding region of the β-globin gene (39, 40). Recently, Hidaka and colleagues (41) have identified an insertional mutation in a splice junction in the human adenine phosphoribosyltransferase gene, causing aberrant splicing. Interestingly, the inserted nucleotide resulting in the $Null_{mattawa}$ allele is a thymidine that occurs at the terminus of a short polymeric sequence of repeating thymidines. This is consistent with the observation in studies of bacteriophage T4 mutants that nucleotide insertion mutations generating frameshifts generally occur in the setting of short repeating polymeric sequences (42–45), a phenomenon which has been explained on the basis of slippage mispairing during transcriptional replication (42, 46). Consistent with this concept, a tetrameric or pentameric polythymidine sequence has been shown to be a frameshift mutational hot spot both in vivo (44, 45) and in in vitro systems evaluating mutagenesis (47, 48).

The insertional mutation of the $Null_{mattawa}$ gene generates a 3′ frameshift with an altered reading frame distal to and including codon 353. This altered reading frame terminates at a premature stop signal at new codon 376, 19 codons upstream from the normal stop signal. Interestingly, the presence of a premature stop signal in the $Null_{mattawa}$ allele is associated with a normal-sized α1AT mRNA transcript. In contrast, the premature stop signal of $Null_{bellingham}$ is associated with the complete absence of detectable α1AT mRNA from α1AT-expressing cells (20). Perhaps an explanation for this difference is that the premature stop signal of the $Null_{bellingham}$ allele occurs near the center of the α1AT mRNA transcript; the lack of α1AT mRNA in this setting may be the consequence of mRNA molecules not protected at the 3′ end by polysomes, as has been proposed for some of the β^0 thalassemia mutations in which no β-globin mRNA is detected (49, 50). In this regard, the $Null_{mattawa}$ allele is associated with a premature stop signal near the terminal part of the α1AT mRNA molecule. In this setting, the occurrence of the stop signal at a distal position may allow sufficient protection by polyribosomes such that a stable α1AT mRNA transcript results.

As the α1AT $Null_{mattawa}$ allele is associated with α1AT mRNA transcripts, it is of interest that no α1AT translation product in association with this allele could be detected from cells with the $Null_{mattawa}$ mutation. In vitro translation analysis of the $Null_{mattawa}$ mRNA transcript demonstrated that it directs synthesis of a truncated α1AT molecule. The coded translation product contains alterations of the primary protein structure predictive of structural instability (51). Presumably, the conformational changes affected by these alterations generate a protein species incapable of secretion. This could occur on the basis of intracellular aggregation within a subcellular compartment or alternatively, secondary to intracellular degradation of the nascent protein. The α1AT $Null_{hong\ kong}$ gene has recently been shown to result in a truncated α1AT protein which aggregates within the cell at the level of the rough endoplasmic reticulum (23). In contrast, not only can no α1AT be detected as a secretion product from α1AT-expressing cells with the $Null_{mattawa}$ gene, but additionally, no detectable intracellular form of α1AT can be identified aggregating within the cell. Thus, it is likely that translation of the $Null_{mattawa}$ mRNA results in synthesis of a structurally unstable, truncated α1AT molecule, with the consequence that the altered α1AT protein is degraded within the cell and thus not present in the serum in detectable amounts.

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