47 (1988)] to ATT by site-directed mutagenesis, to generate pVL941 (V. A. Luckow and M. Summers, in preparation). A polylinker was inserted into the Bam HI site 30 bp 3′ to this ATT to generate pAcC9. Transfer vectors containing clone 101 or clone 16 GAP cDNA were transfected into Sf9 cells and recombinant virus prepared as in Luckow and Summers (1988). Four days after infection, cells were pelleted and extracts made with 0.5% NP-40, 10 m*M* tris-HCl (*p*H 8.0), 150 m*M* NaCl. Extracts were diluted into GAP assay buffer as above. These extracts could be diluted more than 10,000-fold without loss of GAP activity.

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Repair of the Secretion Defect in the Z Form of α1-Antitrypsin by Addition of a Second Mutation

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Homozygous inheritance of the Z-type mutant form of the α1-antitrypsin (α1AT) gene results in the most common form of α1AT deficiency, a human hereditary disease associated with a high risk for the development of emphysema and an increased incidence of neonatal hepatitis. The α1AT-synthesizing cells of individuals with the Z gene have normal α1AT messenger RNA levels, but α1AT secretion is markedly reduced secondary to accumulation of newly synthesized α1AT in the rough endoplasmic reticulum. Crystallographic analysis of α1AT predicts that in normal α1AT, a negatively charged Glu^{342} is adjacent to positively charged Lys^{290}. Thus the $Glu^{342} \rightarrow Lys^{342}$ Z mutation causes the loss of a normal salt bridge, resulting in the intracellular aggregation of the Z molecule. The prediction was made that a second mutation in the α1AT gene that changed the positively charged Lys^{290} to a negatively charged Glu^{290} would correct the secretion defect. When the second mutation was added to the Z-type complementary DNA, the resulting gene directed the synthesis and secretion of amounts of α1AT similar to that directed by the normal α1AT complementary DNA in an in vitro eukaryotic expression system. This suggests the possibility that a human hereditary disease can be corrected by inserting an additional mutation in the same gene.

THE SERUM GLYCOPROTEIN α1-ANTITRYPSIN (α1AT) is produced by hepatocytes and mononuclear phagocytes and is a major inhibitor of the powerful serine protease, neutrophil elastase (*1–4*). The α1AT gene is a 12-kb single-copy gene on chromosome 14 (*5*). The gene is pleomorphic, with 75 variants known (*2*). Between 1 and 2% of the α1AT alleles carried by Caucasians of northern European descent contain the "Z" type α1AT gene, an α1AT variant associated with reduced α1AT serum levels (*1, 2*). When the Z gene is inherited in a homozygous fashion, serum α1AT levels are 3.0 to 5.8 μ*M*, as compared to the normal 20 to 48 μ*M* (*1, 2*). This decrease results in an insufficient amount of α1AT to protect the lower respiratory tract from the ravages of neutrophil elastase, and the individual is at high risk for the eventual development of emphysema (*6*).

The Z α1AT gene differs from the normal M1(Ala^{213}) gene by a single base G → A change in the codon GAG of Glu^{342} that changes the residue to Lys (*7, 8*). The homozygous Z "deficiency" state results from post-translational events occurring in the rough endoplasmic reticulum (RER) of α1AT-producing cells (*9–12*). The α1AT-producing cells of such individuals have normal levels of α1AT mRNA (*13, 14*), but a marked reduction in the secretion of α1AT, and an accumulation of α1AT with high mannose–type carbohydrate side chains in the RER (*15, 16*). Consistent with these observations, liver biopsies of Z homozygotes show aggregation of α1AT in the RER of hepatocytes (*17, 18*). Although the mechanisms of the accumulation of the Z-type α1AT has not been completely elucidated, the crystallographic structure of the protein predicts that Glu^{342} in β-sheet A strand 5 normally forms a salt bridge link with nearby residue Lys^{290} in β-sheet A strand 6 (Fig. 1), thus helping the newly synthesized α1AT molecule to fold into its three-dimensional configuration (*19*). It has been suggested that the inability to form this salt bridge in Z mutants results in the accumulation of α1AT, in other words, the mutation at Glu^{342} impedes normal intracellular processing of the α1AT, and consequently interferes with its secretion (*19*).

We hypothesized that the Z-associated α1AT "deficiency" state might be prevented by reestablishing the salt link through modification of a second residue in the α1AT molecule. Theoretically, this second mutation would reconstitute the charge balance between residues 342 and 290 by changing the negative charge at residue 290 to a positive charge (Fig. 1). Three α1AT gene variants were constructed by means of oligonucleotide site-directed mutagenesis with a full-length human α1AT cDNA: a normal cDNA, a cDNA with the Z mutation, and a cDNA with the critical Z mutation at residue 342 and the additional mutation ($Lys^{290} \rightarrow Glu^{290}$). The cDNAs were inserted into a eukaryote expression vector which was then used to transfect and direct COS I cells (an SV40-transformed African Green monkey kidney line) to transcribe, translate, and secrete the three forms of human α1AT.

As expected, similar levels of α1AT mRNA transcripts were observed in COS I cells transfected with normal, Z, or Z-charge reconstituted (Z-CR) α1AT variants (Fig. 2A). In six experiments, no significant differences were observed in mRNA content ($P > 0.3$) as measured by a one-way analysis of variance in a multiple comparisons procedure. All three variants secreted a protein of normal size (52 kD) that was immunoprecipitated with rabbit antibody to human α1AT and migrated with human α1AT (Fig. 2B). However, the cells transfected with the Z variant cDNA secreted only 26 ± 4% ($\bar{X}$ ± SEM) of the α1AT secreted by the cells transfected with the normal variant ($P < 0.005$). Thus, the single mutation in the Z cDNA variant was sufficient to reproduce the marked reduction in secretion of α1AT comparable to that seen in α1AT-producing cells of individuals homozygous for the Z-type α1AT deficiency. In contrast, when the COS I cells were transfected with the Z-CR variant, the transfected cells secreted 93 ± 15% of the amount of α1AT secreted by cells transfected with the normal cDNA despite the presence of the Z mutation at residue 342 ($P > 0.3$, compared to normal). Thus, the addition of the second mutation at residue 290 obviated the molecular pathology associated with the Z mutation. Although an insufficient amount of the Z-CR protein was available to evaluate its function as an inhibitor of neutrophil elastase, we predict that the function of the protein should not be altered, because the second mutation was placed at a site far

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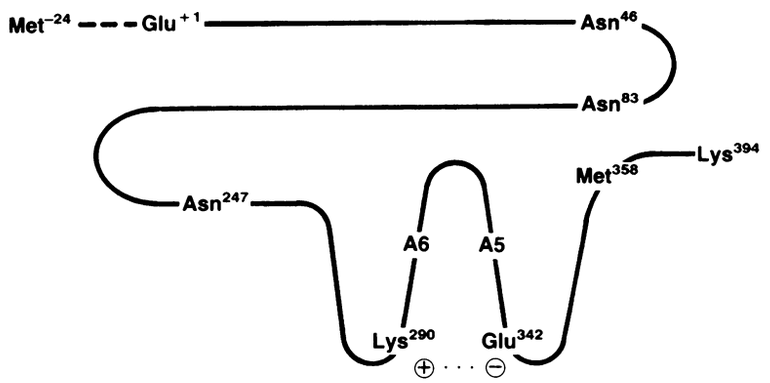
from the active site of the protein (*1–4*).

The fact that the molecular pathology associated with the Z mutation can be reproduced in COS I cells, a cell that does not normally secrete α1AT, supports the concepts that: (i) the intracellular translocation system utilized by α1AT is likely a general protein translocation system common to many secretory eukaryotic cells (*11, 12, 20*); and (ii) the single mutation causing the substitution $Glu^{342} \rightarrow Lys^{342}$ is sufficient to cause the α1AT deficiency state associated with the Z gene (*8, 21, 22*). Consistent with these observations, when α1AT mRNA extracted from liver of homozygous Z individuals is translated by *Xenopus* oocytes, there is a marked reduction in α1AT secretion compared to that directed by α1AT mRNA from normal liver (*11, 12*).

Although the mechanism responsible for the secretion abnormality associated with the Z mutation is not completely understood, the leading hypothesis has been that the resulting change in the charge at position 342 results in misfolding or slowing of the rate of folding of the newly synthesized α1AT molecule in the RER, with consequent local aggregation preventing normal translocation to the Golgi (*19*). However, this cannot be the entire mechanism, since in vitro transfection studies with the normal α1AT cDNA containing $Lys^{290} \rightarrow Glu^{290}$ have shown normal levels of α1AT secretion (*22, 23*). Thus, the consequences of the $Glu^{342} \rightarrow Lys^{342}$ Z mutation may not entirely result from the loss of the salt bridge per se, that is, they may occur because the Z molecule may not be recognized normally by the translocation machinery in the RER. While our study cannot distinguish between these hypotheses, it indicates that the Lys^{342} in the Z protein is the critical residue.

The Z-CR variant is a direct demonstration that the biologic abnormalities of a human hereditary disorder can be prevented by adding a second mutation to the abnormal gene, thus essentially "repairing" the molecular pathology of the hereditary disease. While such an approach does not have immediate obvious application to the treatment of a disorder like α1AT deficiency, it does have direct implications relevant to the clinical disorder. First, there may be individuals with the $Glu^{342} \rightarrow Lys^{342}$ mutation who have a naturally occuring second mutation that "repairs" the Z mutation. Genotypically, these individuals would be Z with respect to residue 342 but, at the protein level, would appear to be identical to normal individuals. Second, while it may not be presently possible to insert a second mutation into the Z gene in vivo, it may be possible to develop pharmaceutical agents that would alter the charge milieu in the RER of α1AT-synthesizing cells such that the dramatic consequences of the $Glu^{342} \rightarrow Lys^{342}$ mutation in the Z gene would be obviated.



Variant	Codon	Amino acid	Charge	Charge	Amino acid	Codon
N	AAA	Lys^{290}	⊕	⊖	Glu^{342}	GAG
Z	AAA	Lys^{290}	⊕	⊕	Lys^{342}	AAG
Z-CR	GAA	Glu^{290}	⊖	⊕	Lys^{342}	AAG

Fig. 1. The wild-type α1AT molecule and the variant forms of α1AT cDNAs used to evaluate the Z mutation. **Top**: Schematic representation of the primary translation product of the α1AT molecule showing the 24-residue signal peptide, Glu^{+1} of the mature protein, the three carbohydrate attachment sites (Asn^{46}, Asn^{83}, Asn^{247}), Lys^{290} at the end of β-sheet A strand 6, Glu^{342} at the end of β-sheet A strand 5, the active site center Met^{358}, and the COOH-terminal Lys^{394} of the mature protein. The Lys^{290} to Glu^{342} salt bridge is indicated by (···). **Bottom**: Variant α1AT cDNAs used to evaluate α1AT synthesis in an in vitro eukaryotic expression system. Variant cDNAs were created by oligonucleotide-directed mutagenesis (*24, 25*). A Bam HI–Pst I fragment of the normal human α1AT cDNA pTG603 (*26*) was subcloned into the single strand phage M13 mp19 and oligonucleotide-directed mutagenesis with 19 bp primers was used to create single base substitutions as indicated. The cDNAs were subcloned into the Bam HI site of the eukarayote expression vector pSVL (Pharmacia). N, normal; Z, Z-type, Z-CR, Z–charge reconstituted.

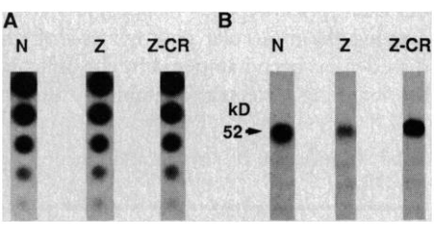


Fig. 2. Evaluation of the α1AT mRNA content and α1AT synthesis by COS I cells transfected with normal, Z, and Z-CR variants. (**A**) α1AT mRNA content. The COS I cells were transfected by calcium phosphate precipitation (*27*) and the cultures continued in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After 72 hours at 37°C, equal numbers of transfected COS I cells were harvested, cytoplasmic RNA was isolated (*28*), and α1AT mRNA levels determined with dot blot analysis with a ^{32}P-labeled human α1AT cDNA probe pPB01. RNA content was quantitated by laser densitometry. Each variant was evaluated in triplicate in each of six separate experiments. Shown is an autoradiogram of one experiment demonstrating serial 1:2 dilutions of RNA. (**B**) Secretion of α1AT. At 72 hours after the initial transfection, equal numbers of transfected COS I cells were washed twice with cold phosphate-buffered saline (PBS), *p*H 7.4, and incubated in DMEM deficient in methionine and supplemented with dialyzed 10% FBS and [^{35}S]methionine (>1000 Ci/mmol, 250 μCi/ml; Amersham) for 60 min, 37°C. The cells were then washed twice with cold PBS and cultured in complete medium for 0 to 2 hours. The α1AT was immunoprecipitated from 1.5×10^4 total tricholoracetic acid precipitable dpm in the supernatants of the transfected cells with a specific human antibody to α1AT (*14*). The immunoprecipitated α1AT was evaluated by SDS-polyacrylamide gels and fluororadiography (*14*) with human α1AT as a standard. A typical fluororadiogram of the α1AT immunoprecipitated from culture supernatants is shown. The amount of α1AT secreted by the COS I cells was quantitated by laser densitometry of the fluorograms. Each variant was evaluated in triplicate in three experiments.

REFERENCES AND NOTES

1. J. E. Gadek and R. G. Crystal, in *α1-Antitrypsin Deficiency*. J. B. Stanbury and J. B. Wyngaarden, Eds. (McGraw-Hill, New York, 1982), pp. 1450–1467.
2. M. Brantly, T. Nukiwa, R. G. Crystal, *Am. J. Med.* **84** (suppl. 6A), 13 (1988).
3. R. W. Carrell *et al.*, *Nature* **298**, 329 (1982).
4. J. Travis and G. S. Salvesen, *Annu. Rev. Biochem.* **52**, 655 (1983).
5. M. Rabin *et al.*, *Somatic Cell Mol. Genet.* **12**, 209 (1986).
6. J. E. Gadek, G. A. Fells, R. L. Zimmerman, S. I. Rennard, R. G. Crystal, *J. Clin. Invest.* **68**, 889 (1981).

7. T. Nukiwa *et al.*, *J. Biol. Chem.* **34**, 15989 (1986).
8. R. N. Sifers *et al.*, *Nucleic Acids Res.* **15**, 1459 (1987).
9. D. M. Errington, I. C. Bathurst, E. D. Janus, R. W. Carrell, *FEBS Lett.* **148**, 83 (1982).
10. I. C. Bathurst, J. Stenflo, D. M. Errington, R. W. Carrell, *ibid.* **153**, 270 (1983).
11. R. C. Foreman, J. D. Judah, A. Colman, *ibid.* **168**, 84 (1984).
12. K. H. Verbanc and E. C. Heath, *J. Biol. Chem.* **261**, 9979 (1986).
13. D. H. Perlmutter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6918 (1985).
14. J.-F. Mornex *et al.*, *J. Clin. Invest.* **77**, 1952 (1986).
15. A. Hercz, E. Katona, E. Cutz, J. R. Wilson, M. Barton, *Science* **201**, 1229 (1978).
16. I. C. Bathurst, J. Travis, P. M. George, R. W. Carrell, *FEBS Lett.* **177**, 179 (1984).
17. H. L. Sharp, R. A. Bridges, W. Krivit, E. F. Freier, *J. Lab. Clin. Med.* **73**, 934 (1969).
18. A. K. Bhan, R. J. Grand, H. R. Colten, C. A. Alper, *Pediatr. Res.* **10**, 35 (1976).
19. H. Loebermann, R. Tokuoka, J. Deisenhofer, R. Huber, *J. Mol. Biol.* **177**, 531 (1984).
20. H. F. Lodish, *J. Biol. Chem.* **263**, 2107 (1988).
21. M. Brantly *et al.*, *Am. Rev. Respir. Dis.* **135**, A291 (1987).
22. R. C. Foreman, *FEBS Lett.* **216**, 79 (1987).
23. A. A. McCracken, K. B. Kruse, J. L. Brown, *J. Cell. Biochem. Suppl.* **12B**, 286 (1988).
24. M. J. Zoller and M. Smith, *DNA* **3**, 479 (1984).
25. R. B. Wallace, M. Schold, M. J. Johnson, P. Dembek, K. Itakura, *Nucleic Acids Res.* **9**, 3647 (1981).
26. M. Courtney *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 669 (1984).
27. F. L. Graham and A. J. Van Der Eb, *Virology* **52**, 456 (1973).
28. B. A. White and F. C. Bancroft, *J. Biol. Chem.* **257**, 8568 (1982).
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Point Mutations in the Human Vitamin D Receptor Gene Associated with Hypocalcemic Rickets

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Hypocalcemic vitamin D–resistant rickets is a human genetic disease resulting from target organ resistance to the action of 1,25-dihydroxyvitamin D_3. Two families with affected children homozygous for this autosomal recessive disorder were studied for abnormalities in the intracellular vitamin D receptor (VDR) and its gene. Although the receptor displays normal binding of 1,25-dihydroxyvitamin D_3 hormone, VDR from affected family members has a decreased affinity for DNA. Genomic DNA isolated from these families was subjected to oligonucleotide-primed DNA amplification, and each of the nine exons encoding the receptor protein was sequenced for a genetic mutation. In each family, a different single nucleotide mutation was found in the DNA binding domain of the protein; one family near the tip of the first zinc finger (Gly→Asp) and one at the tip of the second zinc finger (Arg→Gly). The mutant residues were created in vitro by oligonucleotide directed point mutagenesis of wild-type VDR complementary DNA and this cDNA was transfected into COS-1 cells. The produced protein is biochemically indistinguishable from the receptor isolated from patients.

A GENETIC LESION IN THE PATHWAY of vitamin D action has been suspected in the human disorder of hypocalcemic vitamin D–resistant rickets (HVDRR) (*1*). This rare, autosomal recessive syndrome is characterized by hypocalcemia, secondary hyperparathyroidism, and early onset rickets, all of which develop despite an increase of the calcium regulating hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in the plasma. In many cases total body alopecia is also present but its relation to vitamin D physiology is unknown. This constellation of features results from peripheral target tissue resistance to the hormone in a manner analogous to the clinical disorders of glucocorticoid (*2*), mineralocorticoid (*3*), and androgen resistance (*4*). While numerous patients have been reported with the HVDRR phenotype (*5–10*), progress in unraveling the molecular pathogenesis of this disorder has been hampered by the difficulty in obtaining receptor containing "target" tissue (usually intestine and bone) for which analysis could be performed. However, fibroblasts cultured from human skin have been shown to contain the 1,25-$(OH)_2D_3$ receptor (VDR) (*11*) and to exhibit biological response to this hormone (*9*). Skin fibroblasts from patients and family members with HVDRR have been evaluated, and a spectrum of molecular defects has emerged including decreased or absent 1,25-$(OH)_2D_3$ binding or decreased DNA binding of the VDR (*6–10*). All cases tested show an inability to induce the enzyme 25-hydroxyvitamin D-24-hydroxylase, a useful marker of receptor-mediated 1,25-$(OH)_2D_3$ action (*7–10*).

We have studied two families for clues to their VDR defects (*7, 8*). The first family (the D kindred) is the result of a consanguinous marriage in a black Haitian family and includes nine members, five of whom have been evaluated [(*7*) and our data]. The parents (D4 and D5) are phenotypically normal, as is one unaffected daughter (D3). Two affected daughters (D1 and D2) display severe rickets and the classic childhood phenotype of HVDRR. The second (the G kindred) is a family of Arabs living in the Middle East (*8*). The parents (G3 and G4) are first cousins and are phenotypically normal with no calcium or bone abnormalities. Of the six children, two males (G1 and G2) display the HVDRR phenotype.

Fibroblasts or lymphocytes (or both)

Table 1. Summary of physical and functional properties of vitamin D receptor in two kindreds with HVDRR.

Property	Wild type	D kindred			G kindred	
		Parents*	Unaffected siblings	Affected siblings	Parents	Affected siblings
M (kD)†	48–50	48–50	48–50	48–50	48–50	48–50
N_{max}‡	32 ± 12		41	32; 52	32; 37	20; 32
K_d ($\times 10^{-11} M$)§	5± 2		5	5.5; 6.3	2; 2	2; 3
DNA binding [peak salt elution (*M*)]	0.2	0.1; 0.2	0.2	0.1	0.1; 0.2	0.1
25-hydroxyvitamin D_3-24-hydroxylase activity	+		+	−	+	−

*Cells were Epstein-Barr–transformed lymphoblasts. †Molecular size. ‡N_{max}, in femtomoles per milligram of protein. §Dissociation constant (K_d) for 1,25-$(OH)_2D$; DNA-cellulose binding peak elution, with a monovalent salt; gradient values are ± S.E.

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