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Identification of a Missense Mutation in One Allele of a Patient with Pompe Disease, and Use of Endonuclease Digestion of PCR-amplified RNA to Demonstrate Lack of mRNA Expression from the Second Allele

Nan Zhong, Frank Martiniuk, Stephanie Tzall, and Rochelle Hirschhorn

Division of Medical Genetics, New York University Medical Center, New York

Summary

Infantile-onset glycogen storage disease type II, or Pompe disease, results from a genetic deficiency of the lysosomal enzyme acid alpha glucosidase (GAA). Sequencing of the cDNA from a cell line (GM 244) derived from a patient with Pompe disease demonstrated a T^{953}-to-C transition that predicted a methionine-to-threonine substitution at codon 318. The basepair substitution resulted in loss of restriction-endonuclease sites for *Nco*I and *Sty*I. Analysis of genomic DNA revealed both a normal and an abnormal *Nco*I fragment, indicating that the patient was a genetic compound. *Nco*I and *Sty*I digestion of cDNA, amplified by PCR from reverse-transcribed RNA, demonstrated that >95% of the GAA mRNA in GM 244 was derived from the allele carrying the missense mutation. The missense mutation was uncommon, since it was not detected in 37 additional GAA-deficient chromosomes, as determined by digestion of genomic DNA with *Nco*I and hybridization. The amino acid substitution predicts a new potential site for N-linked glycosylation, as well as major changes in secondary structure of the protein. We could confirm that the mutation was responsible for the enzyme deficiency by demonstrating that a hybrid minigene containing the mutation did not express GAA enzyme activity after transient gene expression. We have therefore now provided the first identification of a single-basepair missense mutation in a patient with Pompe disease and furthermore have demonstrated that the patient is a genetic compound with the second allele barely expressing mRNA.

Introduction

Acid alpha glucosidase (GAA) is a lysosomal enzyme that hydrolyzes alpha 1–4 and 1–6 glycosidic linkages in maltose, isomaltose, glycogen, and the artificial substrate, 4-methylumbelliferyl-alpha-D-glucoside (Hers 1963; Brown et al. 1970; Salafsky and Nadler 1973; Swallow et al. 1975; Martiniuk et al. 1984). The cDNA has been isolated and contains 3,629 bp, including 2,856 bp of coding sequence, 218 bp of 5′ untranslated sequence and 555 bp of 3′ untranslated sequence (Martiniuk et al. 1986, 1990*b;* Hoefsloot et al. 1988).

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Address for correspondence and reprints: Frank Martiniuk, Division of Medical Genetics, New York University Medical Center, 550 First Avenue, New York, NY 10016.

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Genetic deficiency of GAA results in glycogenosis type II, which is inherited as an autosomal recessive trait and results in a clinical spectrum of disease, ranging from a rapidly fatal infantile-onset glycogen storage disorder (Pompe disease) to a slowly progressive adult-onset myopathy. The infantile-onset form is characterized by accumulation of glycogen in cardiac and skeletal muscle, as well as in other tissues. Clinical symptoms usually are manifested during the first months of life, and patients usually die before their second year (Pompe 1932; Courtecuissf et al. 1965; Engel et al. 1973; Mehler and DiMauro 1977; reviewed in Hers et al. 1989). In prior studies, different mutations for this disease have been inferred from differing alterations in biochemical parameters, including measurements of residual enzyme activity and of residual protein as detected by antibody (CRM), as well as from different aberrations in processing (Beratis et al. 1978, 1983; Hasilik and Neufeld 1980;

Hilkens et al. 1981; Oude Elferink et al. 1985; Reuser et al. 1985; LaBadie 1986; Tager et al. 1987; Hoefsloot et al. 1990; Martiniuk et al. 1990*a*). More recently, northern and Southern analysis of mRNA and DNA has more directly demonstrated genetic heterogeneity (Martiniuk et al. 1986, 1990*a*; Van der Ploeg et al. 1989). These studies have revealed both absence or abnormal size of mRNA in a large proportion of patients and altered restriction-endonuclease fragments in a few patients (Martiniuk et al. 1986, 1990*a*; Van der Ploeg et al. 1989). However, the precise molecular basis for the disease has not been reported for any patient.

The cell line studied in the present paper (i.e., GM 244) was derived from a patient with the infantile-onset form (Pompe disease) and has been reported to exhibit a normal 3.6-kb mRNA on northern analysis (Martiniuk et al. 1986) but to lack both enzyme activity and protein as detected by polyclonal antibody (Beratis et al. 1983; LaBadie 1986; Martiniuk et al. 1990*a*). Southern analysis showed a normal restriction-endonuclease pattern for *Eco*RI, *Pst*I, and *Sac*I (Martiniuk et al. 1986, 1990*a*). We have now sequenced the complete coding region from GM 244 and identified a T^{953}-to-C missense point mutation, predicting a $methionine^{318}$-to-threonine substitution and loss of restriction sites for *Nco*I and *Sty*I. We further confirmed that the mutation was responsible for the disease, by demonstrating that a hybrid minigene containing the mutation did not express GAA enzyme activity after transient gene expression. Analysis of both cDNA and genomic DNA revealed that the patient was a genetic compound. Using a PCR-based assay, we could determine that virtually all of the mRNA was transcribed from the allele carrying the missense mutation.

Material and Methods

Cell Lines

Normal (GM 5659) and GAA-deficient cell lines (infantile onset—GM 244, GM 338, GM 248, GM 4912, and GM 3329; juvenile onset—GM 9282; adult onset—GM 443, GM 6314, GM 1464, and GM 1935) were obtained from NIH Human Genetic Mutant Cell Repository (Camden, NJ). Five additional infantile-onset cell lines (WG 173, WG 285, WG 1102, WG 1099, and WG 482) were obtained from the Mutant Cell Repository in Montreal. Four other GAA-deficient cell lines (one infantile onset—CC; three adult onset—KH, NB, and EB) were also studied, as was the mother of an additional infantile-onset patient.

The cell line studied here, GM 244, was derived from a 5-mo-old Mexican-Caucasian female, exhibits deficient GAA activity and is CRM negative (Hasilik and Neufeld 1980; Beratis et al. 1983; LaBadie 1986; Martiniuk et al. 1990*a*; data are also NIH Human Genetic Mutant Cell Repository information).

Preparation of RNA and DNA, Construction of a cDNA Library, and Sequencing of cDNA

RNA and DNA were extracted from cultured cells as previously described (Martiniuk et al. 1990*b*). A cDNA library from GM 244 was constructed in lambda gt11 by using standard methods (Gubler and Hoffman 1983; Young and Davis 1983). Primary platings of the cDNA library were screened, as previously described, by using a randomly labeled GAA full-length cDNA probe (Feinberg and Vogelstein 1983; Martiniuk et al. 1986, 1990*b*). cDNA inserts were subcloned into pUC19 for double-stranded DNA sequence analysis (Chen and Seeburg 1985), by using the dideoxy chain-termination method (Sanger et al. 1977) with Sequenase (US Biochemicals), ^{35}S dATP (Biggin et al. 1983), and a series of GAA specific primers (Martiniuk et al. 1986, 1990*b*).

Amplification of RNA and DNA by PCR

Three pairs of GAA-specific primers were used for PCR amplification of RNA and DNA (Saiki et al. 1985, 1988); they were (1) a pair of primers, in IVS 1 (5′-TGCTGCAGTGCCAGCCGCGGTTGATGTCT) and IVS 2 (5′-CTTGTGAGGTGCGTGGGTGTCGATGTCCA), that amplified exon 2 from genomic DNA containing bp −32 to 546 of the cDNA, as well as 73 bp 5′ and 47 bp 3′ of intron sequence surrounding exon 2 (Martiniuk et al., in press); (2) a pair of cDNA primers that amplified mRNA at bp 394–1946 (H024—5′-AGCTACCCCAGCTACAAGCTGGAGAA; H002, 5′-AAGCCGCAGACGTCGGCCCCGACCAGAG); and (3) a pair of cDNA primers that amplified at bp 394–1480 (H024 and GAA2—5′-CCACCAGGCCAGGGCTGT).

To amplify mRNA, cDNA was synthesized from 1–2 μg total RNA in 50 μl containing 3–6 μg oligo-dT, 0.5 mM of each dNTP, 50 mM Tris-Cl (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 10 mM DTT, 40 units RNasin (Promega), and 8 units AMV reverse transcriptase (Life Science). The reaction was incubated at

41°C for 2 h. Ten microliters from the first-strand synthesis reaction was amplified in 50 μl containing 1 μg GAA-specific primers, 2.5 units *Taq* polymerase (Perkin Elmer–Cetus), 0.01% gelatin, 50 mM KCl, and 0.2 mM each dNTP. DNA was amplified using an initial 4-min, 94°C denaturation step followed by 30 cycles each of which consisted of 1.5 min at 94°C, annealing for 1.5 min at 65°C, and synthesis for 6 min at 72°C, with the last synthesis occurring at 72°C for 10 min in a DNA thermal cycler (Perkin Elmer–Cetus).

For amplifications of exon 2 from genomic DNA, 0.4 μg DNA was amplified in 50 μl containing 1 μg each primer, 0.2 mM each dNTP, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2 mM DTT, and 2.5 units *Taq* polymerase. Thirty cycles were used, each consisting of 1.5 min denaturation at 94°C, 3 min extension at 72°C, and a final extension at 72°C for 10 min.

Cloning and Sequencing of PCR-amplified Fragments

The amplified DNA was recovered from 0.8% low-melting-point agarose gel (BRL) by heating at 65°C followed by two cycles consisting of freezing in a dry ice–ethanol bath and then thawing for 10 min at 37°C, followed by centrifugation in a microfuge at room temperature for 10 min. The pellet was resuspended in 100 μl H_2O, and the freeze-thaw extraction was repeated. The extracted DNA was precipitated with ethanol and resuspended in 44 μl dH_2O, 4 μl was removed for analysis, and the remaining 40 μl was blunt ended with 3 units Klenow fragment of *E. coli* DNA polymerase I (BRL) with 1 mM each dNTP, 50 mM NaCl, 50 mM Tris-Cl (pH 8.0), and 10 mM $MgCl_2$ in a final volume of 50 μl at 41°C for 1 h (recovery was ~50%). The blunt-ended DNA was cloned into pUC19 at the *Sma*I site. Recombinant colonies were isolated and then were analyzed by restriction-endonuclease digestion of "mini prep" plasmid DNA. Recombinant plasmids were amplified and purified according to standard methods (Sambrook et al. 1989). The double-stranded DNA was sequenced as previously described (Martiniuk et al. 1990*b*).

Digestion of DNA with NcoI *or* StyI*, Southern Transfer, and Hybridization*

Either 1 μg amplified cDNA (1.086 kb, from bp 394–1480) or 12 μg genomic DNA was digested with *Nco*I or *Sty*I (New England BioLabs) according to the manufacturer's direction. The digested amplified cDNA was electrophoresed in 2% agarose gel, and the digested genomic DNA was electrophoresed in 1.2% gel. Both were transferred to nitrocellulose filters (Southern 1975) and hybridized with a randomly labeled 0.448-kb *Xma*I-*Xma*I genomic DNA fragment that contained cDNA sequences from bp 693–955 (Martiniuk et al., in press). DNA from an additional 18 patients with glycogenosis type II (10 infantile, one juvenile, and seven adult onset) and from the mother of one infantile patient was digested with *Nco*I and hybridized as above.

Construction of a Hybrid cDNA Minigene Containing the T-to-C Mutation at bp 953

The 5′ coding sequence containing the basepair change at 953 was isolated in a *Sac*I (bp 426)–to–*Sal*I (bp 993) fragment and ligated to the 3′ *Sac*I site of the normal exon 2. An *Sst*II (bp 318)–*Sal*I fragment was then isolated and exchanged for the normal *Sst*II-*Sal*I fragment in a full-length normal cDNA in a minigene construct. The full-length hybrid cDNA was sequenced through the ligation and mutation sites. The normal minigene construct has previously been shown to express GAA activity and consisted of a GAA promoter segment (a 1.8-kb *Xho*I-*Aat*II genomic fragment containing the 5′ untranslated 187 bp of exon 1, plus an additional 5′ 1.5 kb of genomic DNA) ligated, after the addition of *Hin*dIII linkers, to the 5′ ATG of a full-length normal cDNA that included the 3′ untranslated region with the poly (A) addition site and poly (A) tail. This construct was cloned into pUC19 (Martiniuk et al., in press; Tzall and Martiniuk, in press).

Transient Gene Expression and GAA Enzyme Assay

SV40-immortalized GAA-deficient fibroblasts were derived from GM 4912 (NIH Human Genetic Mutant Cell Repository, Camden, NJ), a cell line with no mRNA or enzyme activity for human GAA. Transient gene expression was performed as described elsewhere (Wigler et al. 1978), with 0.6×10^6 cells/100-mm petri dish plated 24 h before addition of 40 μg calcium phosphate–precipitated DNA from either the hybrid minigene cDNA or normal GAA cDNA plasmids. Cells were harvested 48 h after addition of DNA. Fresh cells were harvested with a rubber policemen, washed by centrifugation with PBS, lysed by sonication, and assayed with 4-methylumbelliferyl-alpha-D-glucoside at pH 4.0, as reported elsewhere (Martiniuk et al. 1984).

Results

Studies of mRNA and DNA

We have previously reported that mRNA from GM 244 was grossly normal in size and amount. The restriction-endonuclease pattern of DNA digested with *Pst*I, *Sac*I, or *Eco*RI and hybridized with a partial cDNA was also normal (Martiniuk et al. 1986, 1990*a*). Hybridization with a full-length cDNA of these filters and with an additional *Hin*dIII digest also failed to reveal any gross abnormalities. The patient was also homozygous for the common allele at three RFLPs (*Rsa*I, *Sac*I, and *Hin*dIII) (Tzall et al. 1990*a*, 1990*b*, and in press).

Screening of a cDNA Library from GM 244, and Sequencing of Recombinant Clones

We isolated, subcloned, and sequenced four positive clones from a cDNA library. Their size range was 0.6–1.6 kb, with the 5′ ends at bp 1768, 2247, 2613, or 2811 (Martiniuk et al. 1990*b*). Sequence analysis of all four clones failed to reveal any abnormalities. The only variation was found at previously reported normal polymorphic sites (Martiniuk et al. 1990*b*) (table 1), consistent with all clones deriving from the same allele.

Amplification of a 5′ cDNA Fragment by PCR, and Detection of a T-to-C Transition at bp 953 That Predicts a Met-to-Thr Substitution

A 5′ cDNA fragment (bp 394–1946) overlapping the largest 3′ cDNA clone was amplified from reverse-transcribed RNA by PCR (fig. 1) and was isolated, subcloned into pUC19, and sequenced. Sequencing of this 5′ cDNA fragment revealed a missense transition of a T-to-C at bp 953 (fig. 2). This T^{953}-to-C transition was found in all 12 clones isolated and predicts a methionine^{318}-to-threonine substitution.

Amplification of the First 547 bp of Coding Sequence of Exon 2 from Genomic DNA

To obtain the remaining 5′ cDNA sequence, we utilized intron primers to amplify exon 2 from genomic DNA. The amplified fragment (0.699 kb) contains the initiation ATG, the first 547 bp of the coding region, 32 bp of 5′ untranslated sequence, and portions of IVS 1 and 2 (Martiniuk et al., in press; see Material and Methods). This fragment overlaps the sequence of the 1.55 kb of the 5′ PCR-amplified cDNA fragment described above (fig. 1). Sequencing of three pools of five clones each revealed two different basepairs (T^{324} or C) at the site of a previously reported silent polymorphism (Martiniuk et al. 1990*b*), thus indicating that GM 244 carried two different alleles in the genomic DNA. No other substitution was found in exon 2 from either allele.

Analysis of Genomic DNA from GAA-deficient Cell Lines for the Loss of an NcoI *Site*

The T^{953}-to-C mutation predicts the loss of an *Nco*I and a *Sty*I site. To confirm that GM 244 was a genetic compound with one allele carrying a T^{951}-to-C missense transition whereas the second allele carried a different mutation, we digested genomic DNA with *Nco*I. Southern analysis of *Nco*I-digested genomic DNA from GM 244 revealed a larger-than-normal 3.603-kb band (due to loss of the *Nco*I site), in addi-

Table 1

Normal Nucleotide Polymorphisms in GM 244

Nucleotide Position	Codon		Amino Acid	
	NL[a]	GM 244	NL[a]	GM 244
324	TGC/TGT	TGT/TGC	Cys/Cys	Cys
596	CAT/CGT	CGT	His/Arg	Arg
668	CGC/CAC	CAC	Arg/His	His
1203	CAG/CAA	CAA	Thr/Thr	Thr
2133	ACA/ACG	ACG	Thr/Thr	Thr
2553	GGG/GGA	GGA	Gly/Gly	Gly
3002	ATC/ATT	ATC	Noncoding	
3082	GCC/GCT	GCT	Noncoding	

[a] The more common basepair at the eight definitely polymorphic sites reported by Martiniuk et al. (1990*b*) is given first.

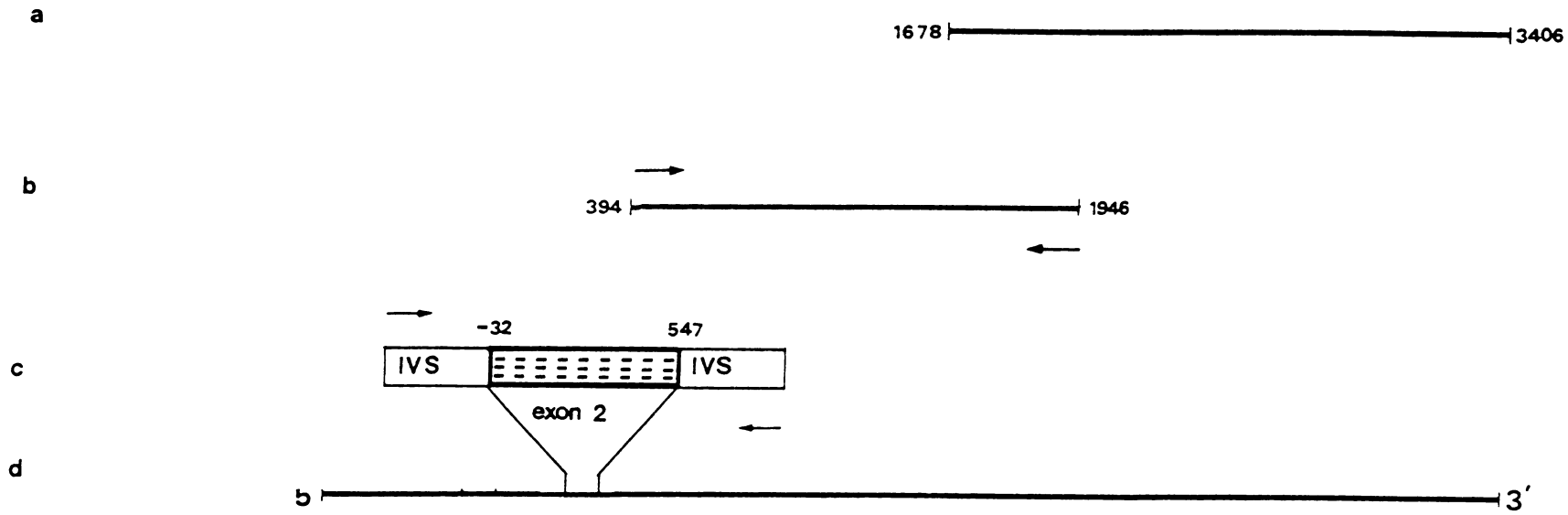

Figure 1 Diagram of cDNA and genomic DNA fragments used for sequence analysis. *a*, 1.638 kb from bp 1768–3406 of cDNA, obtained by screening of cDNA library. *b*, 1.552 kb of cDNA (bp 394–1946 of cDNA), amplified from reverse-transcribed RNA by using GAA-specific primers. This fragment overlapped both the 3′ cDNA (*a*) and the cDNA sequence contained in exon 2 (*c*). *c*, Genomic DNA amplified with intron primers to obtain exon 2 containing bp −32 to 546 of cDNA. *d*, cDNA of GAA bp −32 to 3406.

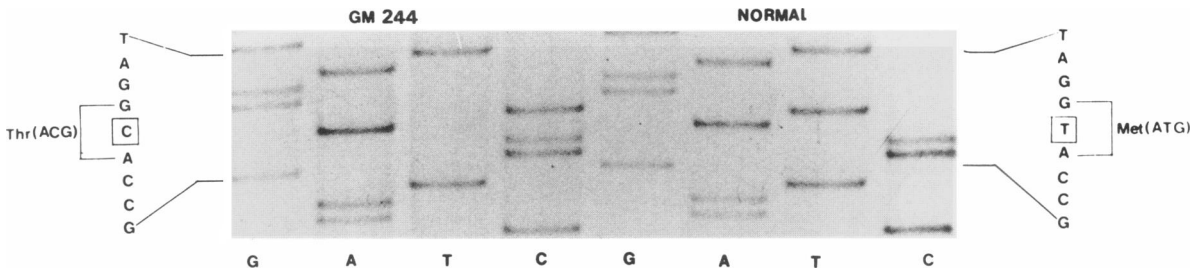

Figure 2 Sequence of Gm 244 and normal. At nucleotide position 953, there was a T-to-C transition in GM 244. This missense transition predicts a methionine^{318} (ATG)–to–threonine (ACG) substitution.

tion to the normal 2.706-kb band predicted on the basis of the sequence of the structural gene (Martiniuk et al., in press). Only the normal 2.706-kb band was seen on analysis of genomic DNA from an additional 37 GAA-deficient chromosomes (from 18 patients, including 10 infantile, one juvenile, and seven adult-onset cases and the mother of an infantile-onset patient) (fig. 3).

Analysis of PCR-amplified RNA for Loss of Restriction-Endonuclease Sites for NcoI *and* StyI

To determine whether mRNA was transcribed from the second allele, we reverse transcribed and amplified a 1.086-kb region and digested it with *Nco*I or *Sty*I (fig. 4). In GM 244, the loss of the *Nco*I site resulted in an abnormal 884-bp band with virtual absence of the normal 558- and 326-bp fragments. Similarly, the loss of the *Sty*I site in GM 244 at position 953 resulted in an abnormal 697-bp band with virtual absence of the normal 371- and 326-bp fragments (fig. 4). With *Nco*I or *Sty*I digestion, these normal bands were also barely detectable in GM 244 after hybridization of Southern transfers with an 0.448-kb genomic DNA probe (not shown). It was estimated that >95% of the mRNA was transcribed from the allele carrying the T-to-C missense mutation.

Analysis of Predicted Secondary Structure of Mutant GAA Protein

The predicted amino acid change of the methionine^{318}-to-threonine substitution occurs two codons downstream of an asparagine and therefore predicts a new site for N-linked glycosylation (Asn^{316}-x-Thr). The amino acid alteration also predicts a major change of protein secondary structure, from an alpha helix to a beta-pleated sheet (fig. 5), when it is analyzed using

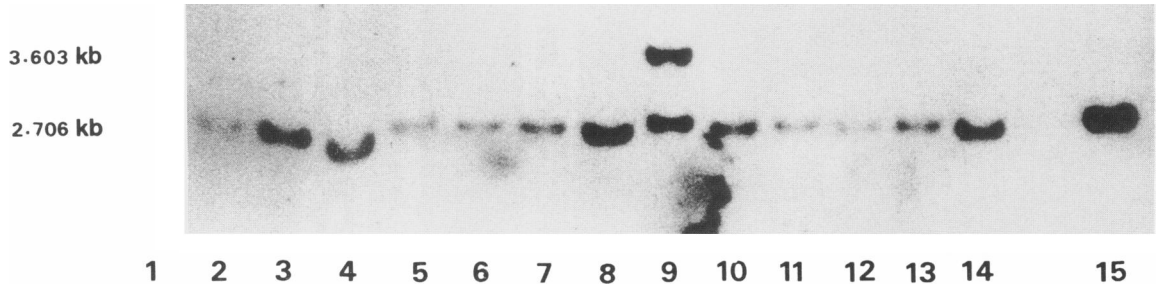

Figure 3 Analysis of genomic DNA. Twelve micrograms of genomic DNA from 18 GAA-deficient patients (10 infantile, one juvenile, and seven adult onset; see Material and Methods), from one parent of an infantile-onset patient, and from one normal was digested with *Nco*I, electrophoresed in 1.2% agarose gel, transferred, and hybridized with a 0.448-kb *Xma*I-*Xma*I genomic DNA probe (bp 693–954 of the cDNA) (Martiniuk et al., in press). Normal DNA (lane 1), from a separate autoradiogram, showed a 2.706-kb band predicted on the basis of the genomic sequence. DNA from GM 244 (lane 9) showed both the normal and an abnormal, 3.603-kb band, resulting from the loss of the *Nco*I site in one allele because of the T-to-C transition at 953 in the cDNA. Twelve patients and a parent are shown (lane 2, GM 338; lane 3, GM 248; lane 4, GM 4912; lane 5, GM 3329; lane 6, GM 443; lane 7, GM 6314; lane 8, GM 1464; lane 10, GM 1935; lane 11, WG 173; lane 12, WG 1102; lane 13, WG 1099; lane 14, WG 482; and lane 15, mother of an infantile-onset patient), and all exhibited only the normal 2.706-kb band. An additional five GAA-deficient patients also showed only the normal 2.706-kb band (see text).

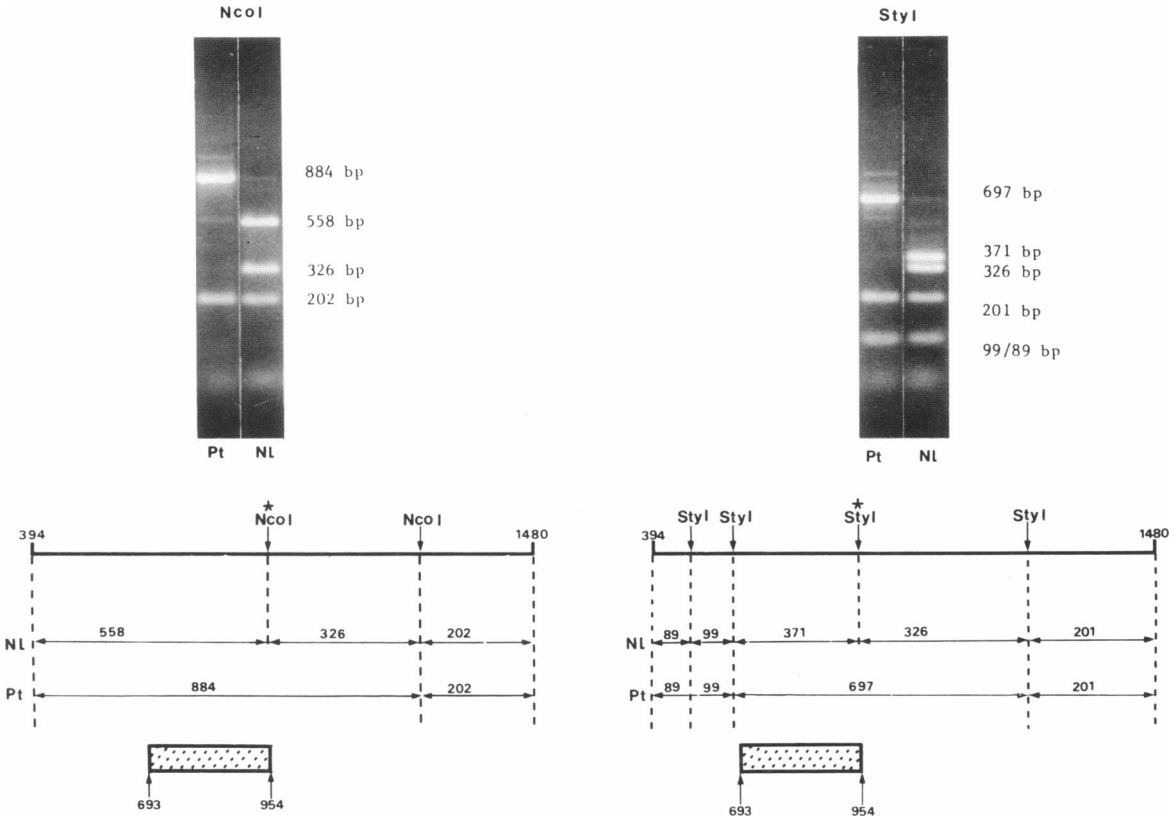

Figure 4 *NcoI* or *StyI* digestion of cDNA amplified from RNA. RNA was reverse transcribed, and the cDNA was amplified by PCR at bp 394–1480. The amplified DNA was digested with *NcoI* or *StyI* and electrophoresed in a 2% agarose gel. (The digested DNAs from the patient and the normal control were electrophoresed at opposite ends of the gel to prevent spillover but, for clarity, have been placed side by side in the figure). *Left, NcoI*: in the normal cDNA (NL), at bp 394–1480, there are two sites for *NcoI* (bp 952 and 1278), resulting in three fragments, one each of 558, 326, and 202 bp. In GM 244 (Pt), an abnormal 884-bp band was generated by loss of the *NcoI* site as a result of the T^{953}-to-C missense mutation. Very faint, normal size 558- and 326-bp bands were also seen in GM 244. The 202-bp band was of normal intensity in GM 244. *Right, StyI*: in the normal (NL) cDNA, there are four sites for *StyI*, one each at bp 483, 582, 953, and 1279, and these result in five fragments, one each of 371, 326, 201, 99, and 89 bp; in GM 244 (Pt), there was a 697-bp band due to loss of the *StyI* site at bp 953. Very faint, normal 371- and 326-bp bands were also visualized in GM 244. The 89/99- and 201-bp bands were of normal intensity.

the Chou-Fasman algorithm (Chou and Fasman 1978), although no such alteration is predicted by the Garnier algorithm (Garnier et al. 1978). In addition, the protein hydrophilicity (amino acids 315–321, with the window of integration for hydrophilicity set to seven residues and a threshold of 1.3), surface probability (amino acids 315–320), and chain flexibility (amino acids 315–319) were increased (Devereux et al. 1984).

Demonstration That the Mutation Causes Loss of GAA Enzyme Activity after Construction of a Hybrid Minigene and Transient Gene Expression

To confirm that the T^{953}-to-C basepair substitution caused a loss of GAA enzyme activity and was therefore responsible for the disease, we constructed a hybrid cDNA by exchanging a 566-bp 5′ segment containing the T^{953}-to-C change for the homologous segment in a normal cDNA, contained in a minigene construct with a GAA promoter segment on the 5′ end (Tzall and Martiniuk, in press; and see Material and Methods). SV40-immortalized GAA-deficient fibroblasts were transiently transfected with this mutant plasmid and with the normal control plasmid and were harvested 48 h later and assayed for GAA activity. Cells transfected with the hybrid cDNA minigene showed no detectable GAA activity (<0.0003 Units/g), while the normal minigene expressed 0.03 Units/g protein GAA enzyme activity. Extracts from untransfected cells had no detectable activity.

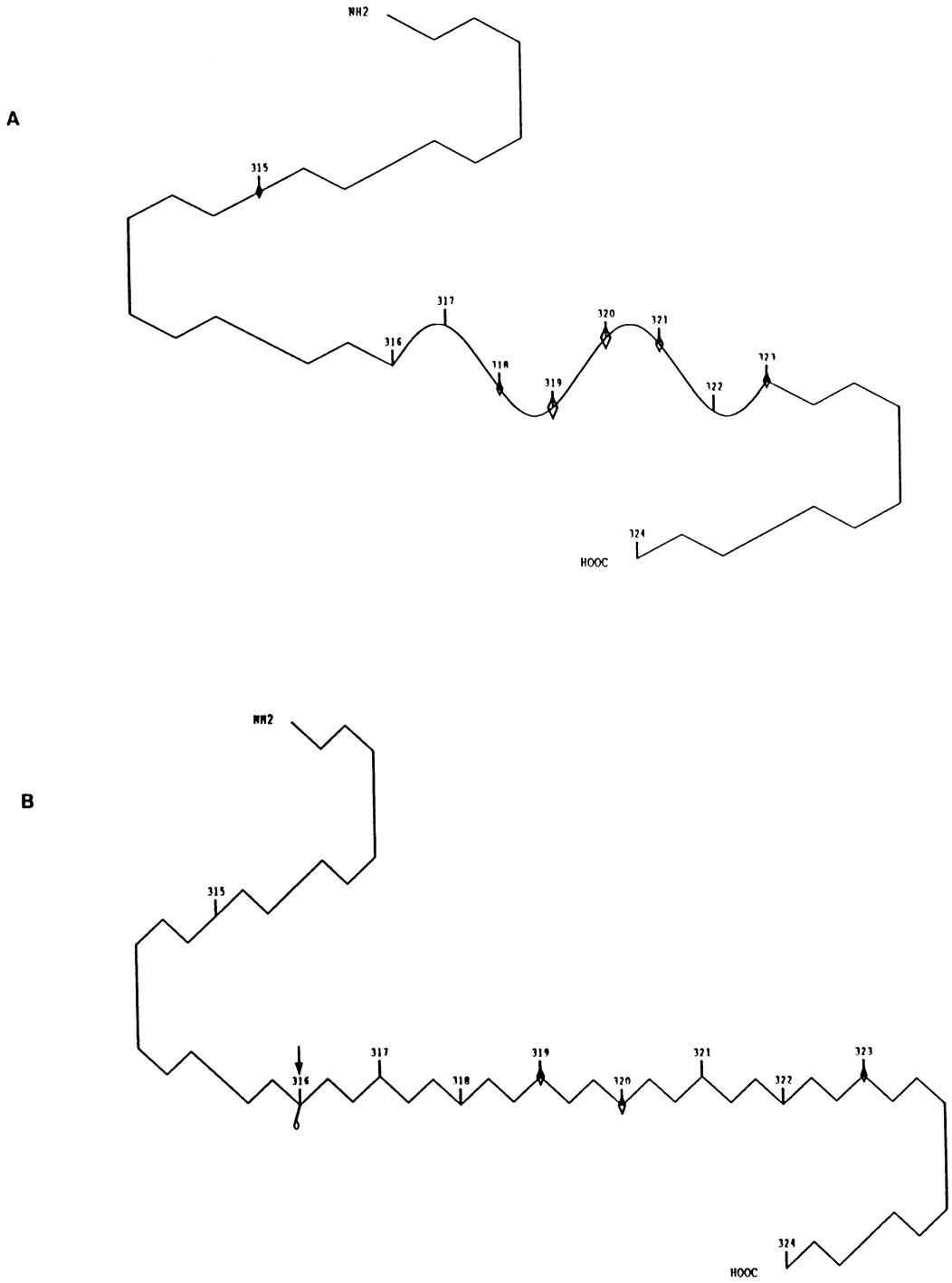

Figure 5 Diagram of predicted protein secondary structure. Predicted secondary structure of GAA analyzed by Chou-Fasman algorithm. The normal (*A*) helix configuration was changed to a beta sheet (*B*) at amino acids 314–323. A new predicted site for N-linked glycosylation is indicated by the arrow. The window of integration for hydrophilicity was set to seven residues and a threshold of 1.3. ○ = KD hydrophilicity ≥1.3; ◊ = KD hydrophobicity ≥1.3.

Discussion

We developed methods to analyze the entire GAA coding sequence and determined the molecular defect in a cell line (GM 244) from a Mexican-American patient with the infantile-onset form of glycogenosis type II. Since we were were not able to obtain a full-length cDNA by standard construction of a cDNA library, we developed methods to amplify the remaining 5′ region of RNA by using PCR. We amplified a 5′ fragment at bp 394–1946 which overlapped the 3′ fragment obtained from the cDNA library. We could obtain the remainder of the 5′ coding region by amplifying exon 2 from genomic DNA that contains the initiation codon ATG and 547 bp of uninterrupted coding sequence and thus overlaps the 5′ amplified cDNA (Martiniuk et al., in press).

These studies allowed us to identify a T^{953}-to-C single-basepair missense mutation, predicting a $methionine^{318}$-to-threonine substitution in one allele of GM 244. We also demonstrated loss of enzyme activity after transient gene expression when the basepair change was introduced into the normal coding sequence. To our knowledge, this is the first identification of a specific mutation in a patient with GAA deficiency. The T^{953}-to-C transition abolishes a site for *Nco*I and allowed us, in addition, to determine, by analysis of genomic DNA, that the patient was a genetic compound. Furthermore, we could determine, utilizing enzyme digestion of PCR-amplified cDNA, that the expressed mRNA was derived almost totally (>95%) from the allele carrying the T-to-C missense mutation. The extremely low steady-state mRNA from this second allele could reflect either an unstable mRNA or a defect in transcription. Further studies are required to determine the precise molecular mechanism accounting for the virtual lack of detectable mRNA from the second allele.

The missense mutation results in a $methionine^{318}$-to-threonine substitution, which predicts major alterations in secondary structure of the protein, consistent with the observed loss of enzyme activity. The amino acid substitution generates a new site for N-linked glycosylation. Both this new glycosylation site (if utilized) and changes in secondary structure could alter the normal antigenicity so that the mutant protein is not recognized by GAA polyclonal antibody. This may explain why there was a normal 3.6-kb mRNA by northern analysis but no cross-reacting material detected by polyclonal antibody (Beratis et al. 1983; LaBadie 1986). Alternatively, the protein could be unstable.

In a survey of 139 single-basepair substitutions causing human genetic disease, one-third of the point mutations were found to occur at CpG hot spots. Ten of the remaining 94 mutations were T-to-C transitions, as is the missense mutation reported here (Cooper and Krawczak 1990). In only one of the 10, in adenosine phosphoribosyltransferase (APRT) deficiency (Hidaka et al. 1988), did the T-to-C transition result in a substitution of ATG (Met) by ACG (Thr), suggesting that the substitution is not a common mutation causing disease. This is consistent with the genetic code, since T-to-C transitions result in amino acid changes at 27 positions (not counting stop to amino acid) but only one of the 27 results in a change of methionine to threonine. Last, DNA from an additional 18 patients showed only a normal *Nco*I fragment, indicating that the mutation is not very common at the GAA locus in the non-Mexican population studied.

Acknowledgments

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