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Leaky Splicing Mutation in the Acid Maltase Gene Is Associated with Delayed Onset of Glycogenosis Type II

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Summary

An autosomal recessive deficiency of acid α-glucosidase (GAA), type II glycogenosis, is genetically and clinically heterogeneous. The discovery of an enzyme-inactivating genomic deletion of exon 18 in three unrelated genetic compound patients—two infants and an adult—provided a rare opportunity to analyze the effect of the second mutation in patients who displayed dramatically different phenotypes. A deletion of Lys-903 in one patient and a substitution of Arg for Leu-299 in another resulted in the fatal infantile form. In the adult, a T-to-G base change at position −13 of intron 1 resulted in alternatively spliced transcripts with deletion of exon 2, the location of the start codon. The low level of active enzyme (12% of normal) generated from the leakage of normally spliced mRNA sustained the patient to adult life.

Introduction

An inherited deficiency of the enzyme acid maltase (acid α-glucosidase [GAA]), an autosomal recessive disorder, leads to lysosomal glycogen storage disease type II (Hers 1963), which can present from infancy to old age, with clinical features that depend on the age at onset (Engel et al. 1970, 1973). If the disease begins in infancy (Pompe 1932; Putschar 1932), hepatomegaly, cardiomegaly, and weakness with hypotonia are noticeable, often at birth, and death from cardiorespiratory failure occurs before the age of 2 years. These clinical findings are caused by the massive accumulation of glycogen in the liver, heart, skeletal muscle, and, to a lesser extent, most other tissues. If the disease begins later—in childhood or any time thereafter—proximal muscle weakness and respiratory insufficiency are the dominant manifestations (Courtecuissf et al. 1965; Engel and Dale 1968; Hudgson et al. 1968; Engel 1970, 1986; Rosenow and Engel 1978). Death usually occurs by respiratory failure.

GAA degrades maltose and glycogen to glucose (Brown et al. 1970; Jeffrey et al. 1970), a catabolic pathway important for the mobilization of glycogen in the neonatal liver (Jezequel et al. 1965). The main function of the enzyme in later life seems to be the prevention of glycogen accumulation in the lysosome. In affected muscle cells, glycogen engorges and disrupts lysosomes. The enzyme is synthesized as a catalytically inactive 110-kD precursor that matures into 76-kD and 70-kD proteins through glycosylation, phosphorylation, and multiple proteolytic modifications (Hoefsloot et al. 1988, 1990*b*; Hermans et al. 1993*c*; Wisselaar et al. 1993).

Biochemical and immunocytochemical studies of affected individuals have detected defects in the synthesis, phosphorylation, and maturation of GAA, but there has been no clear correlation between the biochemical and clinical phenotypes (Reuser et al. 1978, 1985, 1987; Steckel et al. 1982; Beratis et al. 1983*b*; Ninomiya et al. 1984; Miranda et al. 1985). Similarly, Southern- and northern-blot analysis, as well as molecular analysis, demonstrated genetic heterogeneity without defining a genetic correlate to the clinical phenotypes (Martiniuk et al. 1986, 1990*b*; Hoefsloot et al. 1988; Reuser et al. 1988; Hermans et al. 1991*a*; Zhong et al. 1991). Several mutations have been identified in infantile-onset and adult-onset patients (Hermans et al. 1991*a*, 1993*a*, 1993*b*; Martiniuk et al. 1991*b*; Zhong et al. 1991; Huie et al. 1994*a*, 1994*b*; Van der Kraan et al. 1994). A common intron 1 point mutation that results in splicing out of the first coding exon 2 has been recently identified by Huie et al. (1994*b*) in 71% of compound-heterozygous adult-onset patients.

In this paper we describe the genetic defects in three patients from different parts of the world—two infants and one adult. All three have an identical enzyme-inactivating genomic deletion of exon 18 in one allele. Each infant also has a novel inactivating mutation on the second allele. The adult patient has the common intron 1 point mutation on the second allele. We demonstrate that this allele generates three alternatively spliced non-

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functional mRNAs with deletion of exon 2 and a low level of normal transcript, which accounts for the delayed phenotypic expression of the disease. We also present data on alternative splicing of the normal GAA gene.

Patients, Material, and Methods

Patients

A40 is a 42-year-old woman of German extraction who first consulted a physician at age 39 years for progressive proximal muscle weakness and respiratory insufficiency. In retrospect, the patient could recall limitations in her activity as far back as early adulthood. A biopsy of an affected muscle showed a vacuolar myopathy with intracellular deposits of glycogen. GAA activity was 12.3% of normal (2.5 SDs below the mean), and the glycogen content was 5.6-fold greater than normal (18.2 SDs above the mean).

Two primary fibroblast cell lines, WG482 and WG1099, were derived from French Canadian and Dutch male infants who died from Pompe disease. GAA activity was 0.2% and 0.4% of normal, respectively.

Plasmids and cDNA Library

An oligo-dT–primed, unidirectional cDNA library was constructed by Invitrogen using mRNA from an Epstein-Barr virus (EBV)–transformed B-cell line derived from patient A40. The library was screened by standard techniques.

GAA exons 2–20 were removed from puc19-GAA (provided by F. Martiniuk) and were inserted into the pcDNA3 vector, producing the mammalian expression plasmid GAAΔPRO. To generate mutant constructs, we used the recombinant PCR technique (Higuchi 1989). Construct m19 contained the exon 19 mutation identified in patient WG1099; the mutagenized fragment was cloned into *Sse*83871/*Sph*I sites of the expression vector. m5 contained the mutation identified in GAA exon 5 of patient WG482; the mutagenized fragment was inserted between restriction sites *Bpu*1102I and *Sal*I. GAAΔ18 has a deletion of exon Δ18. Two complementary oligonucleotides spanning the deletion were synthesized, annealed, and cloned into the *Sse*8387I/*Kas*I sites of the vector. The mutations and the integrity of the inserts were confirmed by sequencing.

RNA and DNA Isolation for Northern- and Southern-Blot Analyses

Total RNA or mRNA was isolated from cells, as described elsewhere (Aviv and Leder 1972; Chomczynski and Sacchi 1987). The hybridization of northern blots was carried out at 42°C for 36 h in 6 × SSPE, 5 × Denhardt's reagent, 0.5% SDS, 100 μg fragmented salmon sperm DNA/ml, and 50% formamide. After hybridization, the membrane was washed twice with 0.2 × SSC, 0.1% SDS for 15 min at room temperature, followed by two washes at 37°C for 30 min and two washes at 65°C for 30 min.

DNA for Southern-blot analysis was isolated from cells, as described elsewhere (Blin and Stafford 1976). Hybridization and washing of Southern blots were performed as for the northern blots. DNA for PCR analysis was isolated from cells, as described elsewhere (Kawasaki 1990).

PCR Amplification, Denaturing Gradient Gel Electrophoresis (DGGE), and Sequencing

To determine areas of heterozygosity, we used a combination of PCR and DGGE. Genomic DNA was amplified with primers located in the introns and flanking each of the 20 exons of the GAA gene; the length of the flanking intronic sequences varied from 11 to 75 nt (the list of the primers is shown in table 1). Two large exons, exon 2 (578 bp) and exon 20 (606 bp), were amplified in three overlapping fragments. Each of the upstream primers included a 40-bp GC-rich sequence ("GC"-clamp) at the 5′ end, in addition to the specific sequence. Most of the PCR products were obtained by 35 cycles of denaturation at 95°C for 40 s, annealing at 55°C–60°C for 40 s, and extension at 72°C for 60 s. Ten percent dimethylsulfoxide or 1% formamide was added to the PCR mixture when the standard PCR failed to generate the product. Amplification of several exons (2, 5, 15, 11, 18, and 19) required reamplification with nested primers (sequences are available on request). PCR-amplified fragments (~200 ng DNA) were analyzed by DGGE in a Hoefer SE 600 vertical apparatus with the temperature maintained at 60°C, as described elsewhere (Myers et al. 1985; Sheffield et al. 1989). The samples were electrophoresed for 18 h at 80 V on a parallel 6.5% acrylamide gel with a 25%–75% denaturant concentration range for initial screening. The gradient was then optimized, depending on the melting properties of the fragments.

RT/PCR.—Two regions, one spanning exon 2 and one spanning exon 18 were amplified from mRNA. For the exon 2 region, the primers were located in exon 1 (sense: 5′-tgccgcagctgacggggaaactgag) and exon 3 (antisense: 5′-acgcggccgtccagctgccggcg); for exon 18, the primers were located in exon 17 (sense: 5′-cctggacaccatcaacgtcacctcc) and exon 19 (antisense: 5′-ccagctccctcactggtcacacgtac). Either mRNA (2–4 μg) or total RNA (5–10 μg) was transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase. The cDNA (equivalent to 40–80 ng mRNA or 2–4 μg total RNA) was amplified under standard conditions, with an annealing temperature of 55°C and an extension time of 2 min for the exon 2 region and 1 min for the exon 18 region.

Table 1

Primers Used for PCR/DGGE Analysis

Sense[a] (Region)	Antisense (Region)
5′gtgcggaggtgagccgggccggggct (5′ flanking)	5′ggcaggcgcggggcagacgtcag (intron 1)
5′ctgcagtgccagccgcggttgatg (intron 1)	5′ACTGTTCCTGGGTGATGGCCTTGTC (exon 2)
5′CTCACCCAGCTCACCAGCAGGGAGCC (exon 2)	5′TCAGGATGTCCTTGGGGAAGAAGGTGGG (exon 2)
5′GGAACAGTGCGAGGCCCGCGGCTG (exon 2)	5′ggtgtcgatgtccacgcgcaccctc (intron 2)
5′gcgggttgttctctggagagtaaggtg (intron 2)	5′ccctccccatcatgctggcacagagc (intron 3)
5′gctcgtgtggccccttgggtgtgag (intron 3)	5′cagcacctgcgctccccagctcagt (intron 4)
5′ggcactgagctggggagcgcaggt (intron 4)	5′ccctcatgcggacctccagtctcca (intron 5)
5′gggtgcagagccctccaagtgaagaatc (intron 5)	5′cgacttcatgagggagggaggagggagc (intron 6)
5′ccaaggctccctcctccctccctcat (intron 6)	5′ccggccagccccttgcctcccct (intron 7)
5′gctggccgggacgcgtctcctcag (intron 7)	5′cggcccccttcccaaagacccacagt (intron 8)
5′tccccgtgtggcgccagggctctg (intron 8)	5′ggcctctggctttctaacccccgt (intron 9)
5′tggggcttccatgcaggccctggg (intron 9)	5′agtccccagaggccttggggatgctca (intron 10)
5′ccccacccctcctcactctgggcaga (intron 10)	5′tctgatttgattaagtccccagggtag (intron 11)
5′ggcaccttggagcctgccgggagga (intron 11)	5′gacaggctgtgagggcagagccagtgg (intron 12)
5′ggttcccgagtgaccccgctccacacag (intron 12)	5′tctactctgctgagcagcccctcctg (intron 13)
5′ggcctgagctggctctgctgcagca (intron 13)	5′tgggtgggatcgcccacctgccatg (intron 14)
5′ccaggggaccgcggccccagcac (intron 14)	5′tccccccagggcacacatggccac (intron 15)
5′actgagccagccccatcccattcatca (intron 15)	5′ctcccgtctccccagggcttagggtc (intron 16)
5′ctgaggacagcatgggggcctcggca (intron 16)	5′tgtgctgtccacacccccaccatag (intron 17)
5′gccctggaggcctccacctccacca (intron 17)	5′ccaggtcccctcacccctctcaac (intron 18)
5′ccagctgtctgctgacacctcca (intron 18)	5′accttctgccccctgcaccacggctg (intron 19)
5′tgagcgctggggtctcactgctgctg (intron 19)	5′CAGCCCTCCAGGCCCCGGCCCACA (exon 20)
5′GGCTCTGGCCCCCAACGTGTCTAGG (exon 20)	5′GGCATCCAGCCCTCTGGGCAGCCGG (exon 20)
5′CCTGAGCTCCTGCTTCGCGCCT (exon 20)	5′acatcctccctgccctgaatcccag (3′ flanking)

[a] Each primer has a GC-clamp attached to the 5′ end.

Sequencing strategy.—Plasmid DNA was sequenced with Sequenase 2.0 (U.S. Biochemical) according to the manufacturer's instructions. For direct sequencing of the PCR products, two methods were employed: (1) a DNA cycle sequencing system (Promega) as described by the manufacturer and (2) direct sequencing of single-stranded DNA (Nichols and Raben 1994). In each case, both DNA strands were sequenced. To resolve areas of ambiguity or compression on the gels (in particular the GC-rich regions), the sequencing reactions were performed with dITPs instead of dGTPs.

Cell Culture, Transfection, and GAA Assay

Human fibroblast cell lines were obtained from the Mutant Cell Repository in Montreal. TR4219 (provided by F. Martiniuk), is an SV40-immortalized human fibroblast cell line that has no detectable GAA mRNA or enzymatic activity (Martiniuk et al. 1990*a*). B-cell lines were established by transformation with EBV.

TR4219 fibroblasts were cotransfected (calcium phosphate method) with a GAA expression construct (10 μg/10-cm dish) and with human growth hormone (hGH) plasmid (pXGH5) (2 μg/10-cm dish). The GAA activity in the extracts was measured by conversion of the substrate 4-methylumbelliferyl α-D glucoside to the fluorescent product umbelliferone (Barsy et al. 1972; Galjaard et al. 1973; Mehler and DiMauro 1977). A parallel assay for neutral maltase activity was run on each extract, as control for the quality of the extract (substrate: 4-methylumbelliferyl α-D glucoside) (Galjaard et al. 1973; Mehler and DiMauro 1977), and the GAA activity levels were adjusted accordingly. The GAA activity levels were also adjusted for the efficiency of transfection, by measuring hGH in the medium by using reagents supplied by Nichols Institute Diagnostics (Selden et al. 1986).

Results

GAA Exon 18 Deletion (Δ18)

A Southern-blot analysis of genomic DNA from patient A40 revealed that she is a GAA compound heterozygote and that one GAA allele has an alteration near its 3′ end, consistent with a deletion of ~500 bp spanning the *Sac*I site in intron 17 (fig. 1*B*). The region was amplified using flanking PCR primers. Two products were made: one equivalent to that seen in the control and one ~550 bp smaller. Sequencing of these products showed that the larger was identical to the published GAA sequence (Martiniuk et al. 1991*a*) and that the smaller had a 536-bp deletion extending from intron 17 to intron 18 and encompassing exon 18 (fig. 1C). Be-

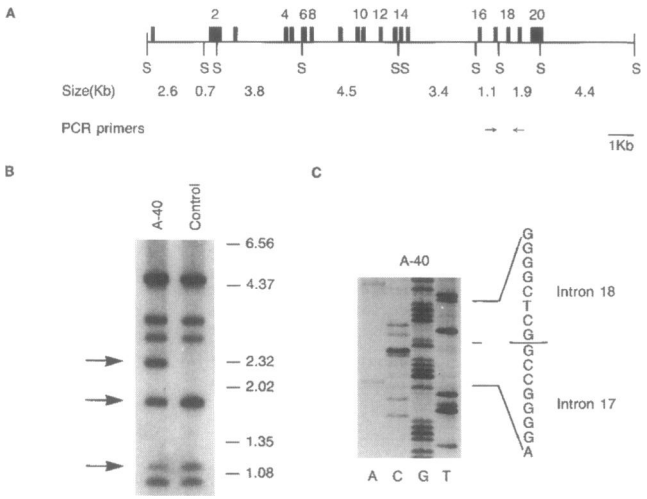

Figure 1 Identification of the exon 18 deletion in a GAA allele of A40. *A*, Diagram of GAA genomic locus (adapted from references (Hoefsloot et al. 1990*a*; Martiniuk et al. 1991*a*). S = predicted *Sac*I endonuclease sites. The sizes (in kb) of the predicted *Sac*I fragments are noted below the diagram. *B*, Southern blot of A40 genomic DNA digested with *Sac*I and probed with GAA cDNA (exons 2–20). Compared with the control, A40 has an additional 2.4-kb band (→) and a reduction in intensity of the 1.9- and 1.1-kb bands by half (→). The numbers to the right of the blot are the molecular weights (in kb). *C*, Sequence of PCR-amplified product obtained by using the primers (→ ←) shown in *A*. The sequence defines a deletion of 534 bp encompassing exon 18.

cause this deletion is bounded by 8-bp direct repeats and deletes one repeat, the breakpoints for the deletion cannot be precisely defined (either 339 or 331 bp upstream of exon 18 and either 31 or 39 bp downstream of exon 18). Analysis of genomic DNA from other family members also detected this deletion in the father and the affected sister.

We have identified the identical mutation in two heterozygous infantile-onset patients, WG482 and WG1099. These two cases were previously studied by Martiniuk et al. (1990*b*), who reported abnormal *Sac*I fragments, and recently Huie et al. (1994*a*) reported the sequence of the exon 18–deleted genomic region. Since GAA activity was negligible in the infants, it was clear that the Δ18 allele would generate nonfunctional product. Thus, an identical and completely inactivating mutation was found in three unrelated patients—two infants and one adult.

GAA Transcription

Sequencing of the RNA/PCR fragments spanning exon 18 in each of the three individuals demonstrated the presence of two products in approximately equal yield—one containing exon 18 and the other with exon 18 deleted, indicating that both alleles are transcribed. Northern-blot analysis of mRNA from A40, WG482, and WG1099 revealed mRNA bands that were smaller

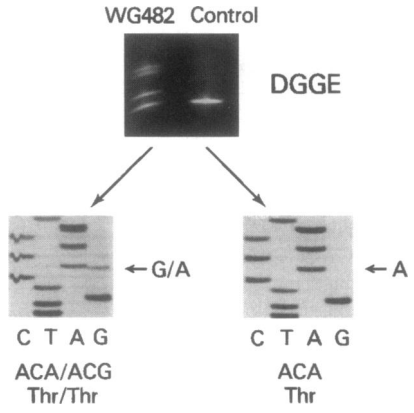

Figure 2 DGGE pattern of the polymorphic exon 15 in the infantile patient. The samples were electrophoresed on a parallel gel, with a denaturant gradient of 25%–75%. The presence of additional bands in the gel, resulting from the formation of heteroduplexes, indicates heterozygosity; the data were confirmed by sequence analysis, showing a silent G-to-A substitution at position 2133.

than the control (not shown). A normal sized transcript was not detected in any of the patients. Furthermore, the asymptomatic parents of individual A40 showed no bands the size of the control, suggesting that the variability in mobility of the GAA mRNA precludes the detection of small size differences by northern-blot analysis.

Delineation of Mutations on the Second Allele

Mutations in the patients with Pompe disease.—DGGE analysis showed that WG482 is heterozygous for exons 5, 11, and 15. Exons 11 and 15 contain previously reported silent substitutions at positions 1581 (A/G) and 2133 (A/G), respectively (Martiniuk et al. 1990*c*). The DGGE pattern of the polymorphic exon 15 is shown in figure 2. Exon 5 is heterozygous for a "T"-to-"G" transversion resulting in a substitution of Arg for Leu-299 (fig. 3). WG1099 is heterozygous for exon 19. A deletion of 3 nt (bases 2706–2708) results in a removal of Lys-903 (fig. 4); the mutation was inherited from the father.

Transfection of TR4219 cells (an immortalized GAA-deficient human fibroblast line) with the pcDNA3 ex-

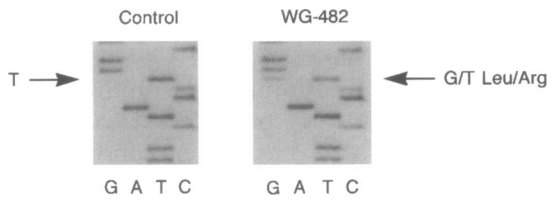

Figure 3 Identification of a missense mutation in GAA exon 5 of patient WG482. Sequencing of a PCR product from genomic DNA showed that one GAA allele has a T-to-G transversion at position 896. This mutation causes the substitution of Arg for Leu-299.

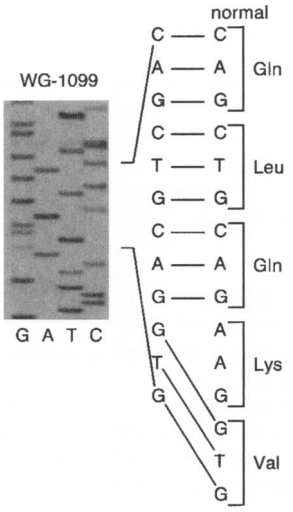

Figure 4 Identification of a 3-nt deletion in GAA exon 19 of patient WG1099. Sequencing of a cloned PCR-amplification product from genomic DNA defined a deletion of guanine-adenine-adenine (bases 2706–2708) from one GAA allele. This mutation deletes lysine-903.

pression vector containing the newly recognized mutations resulted in a complete loss of GAA activity (fig. 5). The results were normalized for transfection efficiency, cell number, and lysis efficiency. The deletion of exon 18 (GAAΔ18), the substitution of Arg for Leu-299 (m5), and the deletion of Lys-903 (m19) are enzyme-inactivating mutations.

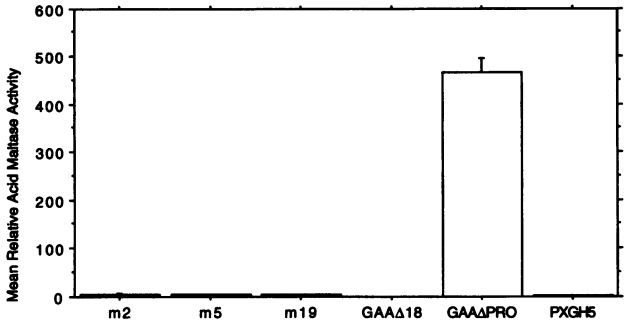

Figure 5 Enzymatic activity of GAA mutants: comparison of the enzymatic activity of the GAA mutants to that in a control. Each GAA mutant was inserted into the pcDNA3 vector and transiently transfected into a human fibroblast line, TR4219, which has no intrinsic GAA activity, along with pXGH5 expressing human growth hormone. The GAA activity was measured in the cell lysate. The results are adjusted for transfection efficiency and are normalized to equivalent neutral maltase activity (relative acid maltase activity). Each result is the average of several independent experiments. The error bar equals 1 SD from the mean. M2 = exon 2 deleted; M5 = T896→G; M19 = bases 2706–2708 deleted; GAAΔ18 = exon 18 deleted; GAAΔPro = complete coding sequence—exons 2–20; and PXGH5 = TR4219 transfected with only pXGH5. Normal fibroblasts have a corrected acid maltase activity approximately twofold higher than does GAAΔ-Pro (data not shown).

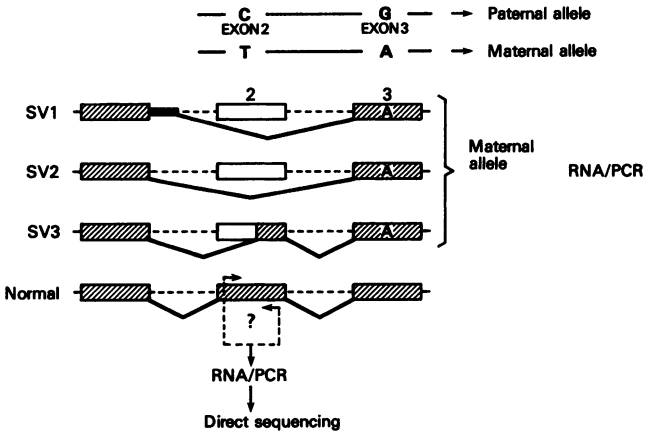

Figure 6 Diagram showing the origin of the splice variants identified in the adult patient. To define the origin of the normal RT/PCR product, exon 2 was amplified from mRNA, by the primers indicated, and was directly sequenced.

Genetic defect in an adult patient.—The PCR/DGGE analysis revealed multiple polymorphic regions spanning exons 2, 3, 5, 8–10, 15, 17, and 20, and failed to localize the defect on the second GAA allele in A40. To identify a mutation, we screened a cDNA library constructed from an EBV-transformed B-cell line derived from the patient. Six clones were isolated. Of these six, five were from the allele with Δ18. The remaining clone was a splicing mutant with a deletion of exon 2 but containing exon 18. In this clone a cryptic consensus splice-donor site in intron 1 (36 nt downstream from the 3′ end of exon 1) is spliced to the acceptor site of exon 3. The rest of the clone had a normal sequence. To confirm that this clone was genuine and not an aberrance, the region spanning exon 2 was amplified from mRNA, and PCR products were isolated and sequenced. Unexpectedly, four products were found: (1) a splice variant that was identical to that isolated from the cDNA library (SV1), (2) a splice variant that spliced from the reported splice-donor site of exon 1 to the acceptor site of exon 3 (SV2), (3) a splice variant that spliced from exon 1 to a cryptic site in exon 2 and then from the splice-donor site of exon 2 to the splice-acceptor site of exon 3 (SV3), and (4) a normally spliced product containing exon 2 with the start codon (table 2 and fig. 6).

It was clear that SV1 was derived from the maternal allele, since it contained exon 18 in the library clone, but the origin of the other products was not obvious, since all library-derived clones from the paternal allele (Δ18) stopped short of the exon 2 region. To elucidate the origin of the splice variants, we took advantage of the presence of the polymorphic site in exon 3. The distribution of the polymorphisms indicates that G-596 in exon 3 in the patient resides on the paternal allele, since the mother is homozygous for an A at this site.

Table 2

Splice Sites Regulating Inclusion of Exon 2 in A40 cDNA

Product[a]	cDNA	Exon 2		Exon 3 Splice Acceptor
		Splice Acceptor	Splice Donor	
Normal	GAGCGGgtgaga	ccgcagGCCTGT	TTCACGgtgggc	ttctagATCAAA
SV1	CCGGCGgtaaca			ttctagATCAAA
SV2	GAGCGGgtgaga			ttctagATCAAA
SV3	GAGCGGgtgaga	cccaagGACATC	TTCACGgtgggc	ttctagATCAAA

NOTE.—The nucleotide sequence found in the cDNA is noted in capital letters. The flanking sequences are listed in small letters (Hoefsloot et al. 1990; Martiniuk et al. 1991); see fig. 7.

[a] Normal = GAA RNA splicing that follows the published exon-intron boundaries; SV1 = aberrant GAA RNA splicing from a cryptic spice-donor site in intron 1 to the splice-acceptor site of exon 3; SV2 = aberrant GAA RNA splicing from the exon 1 splice-donor site to the exon 3 splice-acceptor site; and SV3 = aberrant GAA RNA splicing from the exon 1 splice-donor site to a cryptic acceptor site in exon 2 and then from the published exon 2 splice-donor site to the splice-acceptor site of exon 3.

All of the splice variants are derived from the maternal allele, since they contained A-596, as shown either by sequencing or by restriction digestion with *Nla*III.

To identify a mutation that could account for the deletion, we sequenced the following genomic regions around exon 2: (1) exon 2 and its splice sites—75 bp upstream and 35 bp downstream; (2) the region spanning the cryptic site in intron 1; and (3) exon 1 in the 5′ UTR. The patient is heterozygote for a T-to-G base change in intron 1 at position −13. This mutation has been recently identified by Huie et al. (1994*b*), in 71% of compound-heterozygous adult-onset patients; it resulted in splicing of exon 2 from the transcript and predicted a loss of *Mbo*II site. Restriction digestion with *Mbo*II showed that the mutation in A40 was inherited from the mother (not shown).

We also identified both a deletion of a T at position −23 in intron 1 and a 4-bp insertion in exon 1, which created an upstream in-frame ATG, followed by two stop codons. The deletion and the insertion were on both alleles in the patient and were also found in the control (shown for the insertion in fig. 7), indicating that, most likely, these represent errors in the published sequence.

Deletion of exon 2 from the transcript resulted in a complete loss of enzyme activity in transfection experiments (fig. 5). Thus, the mutations identified in infantile- and adult-onset patients all resulted in generation of inactive proteins, and the question remained as to what accounted for the low level of enzyme activity (12%) in the adult patient A40.

Mutation in intron 1: leakage of normal transcript.—To determine if normally spliced product (containing exon 2) originates from both alleles, we took advantage of another polymorphic site in exon 2. The patient and the parents are heterozygous for C/T at position 324 in exon 2. Sequencing of the cloned PCR fragment (spanning both polymorphic sites in exons 2 and 3) amplified from the father's genomic DNA showed that G-596, which resides on the paternal allele, is in phase with C-324 (fig. 6, *top*). Thus, C-324 in exon 2 is a marker for the paternal allele, and T-324 is a marker for the maternal allele. The RT/PCR product amplified with primers in exon 2 and spanning the polymorphic C/T-324 was sequenced both directly and after subcloning. Both C and T were identified by direct sequencing. Of the 43 clones screened with allele-specific probes, only 4 contained T (maternal allele), and the remaining 39 clones had C (paternal allele) (fig. 8). The data indicate, therefore, that the maternal allele gives rise to a number of mRNAs: (*a*) a low-abundance (~10%) message that includes exon

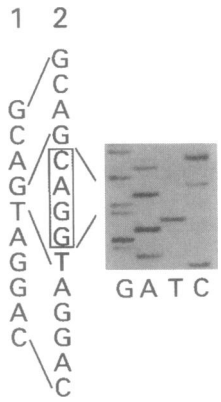

Figure 7 Partial sequence of exon 1 of the GAA gene. 1 = Published sequence (Martiniuk et al. 1991*a*); and 2 = 4-nt insertion identified in A40 and in a normal individual, suggesting that this nucleotide stretch may have been missed in the published sequence of GAA exon 1. Another area of discrepancy in exon 1 is located 40 nt upstream from the 3′ end of the exon: the sequence GGCC was CGCG in the patient and in controls.

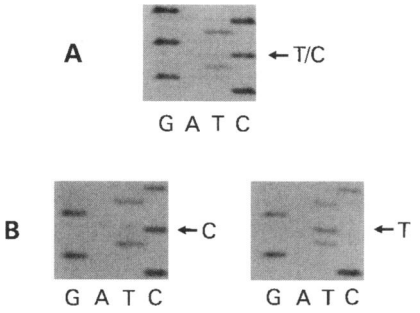

Figure 8 Sequence analysis of the nonmutant RT/PCR product spanning exon 2 in A40. The "T" is a marker for the maternal allele, and the "C" is a marker for the paternal allele. *A*, Direct sequencing of the PCR product. The relative intensity of the T and C suggests that the steady-state level of exon 2 mRNA from the maternal allele is ~10% of that in the paternal allele. *B*, Sequence of the subcloned fragments; 39 clones contained C, and 4 clones contained T.

2 and (*b*) more abundant messages in which exon 2 is spliced out. Small amounts of the variants SV2 and SV3 were also detected in EBV-transformed normal B-cells by Southern-blot analysis of the cDNA amplified with primers located in exon 1 and exon 3 (fig. 9, *middle section*). Both the position of the probes specific for each of the splice variants and the expected size of the products are shown in the upper section of figure 9. The finding was confirmed by sequencing (shown for SV2 in fig. 9); to obtain enough material for sequence analysis, it was necessary to gel-purify the DNA (which was not visible by ethidium bromide staining) and reamplify with nested primers. It is likely that the alternatively spliced forms in normal tissue may have been missed in previous studies, because of their low abundance. In contrast, they constitute a significant proportion of the mRNA in A40, and it is apparent that their relative abundance is much greater in the patient than in the control. Thus, the mutation in the intron 1 results in the generation of aberrantly spliced transcripts, but a low level of normal mRNA is also transcribed from this allele. The leakage of normal transcripts must be responsible for the low level of GAA activity, which has allowed the patient to reach adult life before becoming symptomatic.

To determine the genetic background against which the intron 1 mutation occurred, we sequenced the polymorphic exons identified by the DGGE and the cDNA clone derived from the maternal allele. Table 3 contains the information on the phase at all the polymorphic sites identified in A40.

Discussion

Generally, infantile-onset patients with type II glycogenosis have very low or no detectable enzyme activity, while adult-onset patients have higher enzyme activity. However, residual GAA activity has been considered an incomplete explanation for delayed onset of disease, because there is overlap in the reported GAA activity in patients with infantile-, juvenile-, and adult-onset disease (Shin-Buehring et al. 1978; Beratis et al. 1983*a*, 1983*b*; Ninomiya et al. 1984). Although the genetic background against which a GAA mutation is expressed could slow the expression of a mutant phenotype in patients with and without residual GAA activity, in the few studies that have addressed this possibility no correlation has been found between the age at onset and the

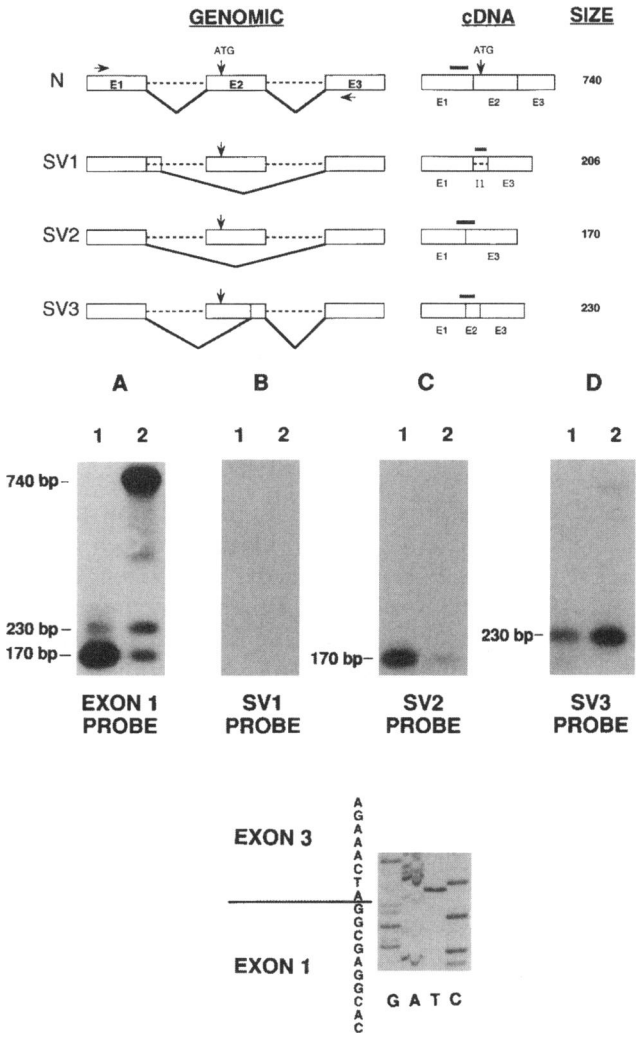

Figure 9 Identification of splice variants in normal B-cells. *Upper section*, Splicing pattern identified in A40. The position of the oligonucleotides used to amplify the region around exon 2 (→ ←) and to probe Southern blots (–), as well as the expected size of the products, are indicated. *Middle section*, Autoradiograms of Southern blots hybridized with (*A*) exon 1 probe, specific for the normal transcript and all three splice variants; (*B*) intron 1 probe specific for SV1; and (*C* and *D*) junctional probes specific for SV2 (*C*) and SV3 (*D*). Lanes 1 and 2 represent two independent RT/PCR reactions. *Lower section*, Direct sequencing of SV2.

Table 3

Normal Polymorphisms in Patient A40

Nucleotide Position	Exon	Codon M/P**	Amino Acid
324	2	TGT/TGC	Cys/Cys
596	3	CAT/CGT	His/Arg
668	3	CGC/CAC	Arg/His
1203	8	CAG/CAA	Gln/Gln
2133	15	ACA/ACG	Thr/Thr
2338	17	GTA/ATA	Val/Ile
3277	20	CTG/CTT	Noncoding

NOTE.—Heterozygosity in exons 5, 9, and 10 was due to base changes in the intronic sequences: g/a in intron 5, 12 nt downstream from exon 5; g/a in intron 8, 18 bp upstream from exon 9; and g/c in intron 9, 19 bp upstream from exon 10.

activity of other glycogenolytic enzymes such as neutral maltase (Soyama et al. 1977; DiMauro et al. 1978; Shanske et al. 1984). Furthermore, biochemical analyses of peptide synthesis, glycosylation, phosphorylation, and proteolysis in many patients have failed to detect a consistent correlation between phenotype and biochemical abnormalities, although abnormalities of each process have been found (Barsy et al. 1972; Murray et al. 1978; Reuser et al. 1978, 1985, 1987; Steckel et al. 1982; Beratis et al. 1983*a*, 1983*b*; Ninomiya et al. 1984; Miranda et al. 1985).

Two adult-onset patients with unusually low (almost negligible) GAA activity in fibroblasts have been studied for the genetic defects (Martiniuk et al. 1991*b*; Hermans et al. 1993*a*, 1993*b*). In one of the patients no mRNA was detected from one GAA allele, which contained a mutation involving exon 18—the substitution of a stop codon for Arg-854. The second allele in this patient had an A-to-C-1935 transversion, causing the substitution of Glu for Asp. This mutation inhibited transport of the 110-kD precursor from the rough endoplasmic reticulum and, consequently, its maturation to an active enzyme. In the second patient, there were two point mutations—in exons 14 and 15—that impaired the intracellular transport and maturation of the enzyme (Hermans et al. 1993*b*).

An in-frame deletion of exon 10, containing the sequence of the catalytic site, has been recently reported in an adult-onset patient (Huie et al. 1994*b*). The patient was also heterozygous for a −13 T-to-G base change in intron 1, which was present in heterozygosity in 71% of adult-onset patients of different ethnic and racial backgrounds.

In the present study, the recognition that a single mutation, Δ18, was common to one allele from each of three patients of apparently different ancestry allowed us to analyze the effect of the second mutation on the phenotypic expression of the disease. Δ18 itself is an in-frame mutation that deletes 55 amino acids and completely inactivates GAA. While the manuscript of the present article was being reviewed, Van der Kraan et al. (1994) showed that the deletion of exon 18 is a frequent mutation in this disease. Huie et al. (1994*a*, 1994*b*) reported a Δ18 mutation in several patients with adult and infantile onset of the disease. Previous studies have shown that the amino acids encoded by exon 18 are crucial for the maturation of GAA, because this exon contains the proteolytic cleavage site for the production of the 76-kD enzyme from the inactive 95-kD precursor (Wisselaar et al. 1993). We presume therefore that this deletion causes a loss of GAA activity in both the adult and the infants by blocking the maturation of GAA. The synthesis of mRNA from this allele appears unaffected in all three patients.

In the cells from the French Canadian infant (WG482), T-896-to-G transversion in exon 5 inactivates GAA through a substitution of Arg for Leu-299. The exact mechanism of inactivation remains unknown, but no known proteolytic sites or glycosylation sites are involved, and this region is not thought to lie in the active site (Hermans et al. 1991*b*, 1993*c*; Wisselaar et al. 1993). In another patient with infantile-onset glycogenosis, a different mutation in exon 5—i.e., T-953-to-C transition—also inactivates GAA (Zhong et al. 1991); no GAA protein, either precursor or mature enzyme, could be detected with polyclonal antisera.

In the cells from the Dutch infant (WG1099), a 3-nt deletion (bases 2706–2708) in exon 19 causes a deletion of Lys-903 and the inactivation of GAA. Again, the mechanism by which this mutation inactivates GAA is unclear, since no known proteolytic sites or glycosylation sites are involved, and this region is not thought to lie in the active site (Hermans et al. 1991*b*, 1993*c*; Wisselaar et al. 1993). Furthermore, this region is proteolytically removed during the maturation of the enzyme (Wisselaar et al. 1993). Therefore, Lys-903 is not required for the functioning of the mature enzyme but is probably involved in formation of a feature of the secondary structure necessary for maturation.

In the adult patient, who had reached nearly her 5th decade before a progressive myopathy and secondary respiratory failure overtook her, we may reasonably presume that, as in the other patients, no functional enzyme was made from the Δ18 allele and that her survival must have been due to the small amounts of normal enzyme generated from the second (maternal) allele, which contained the −13 mutation. The major products from the maternal allele in the patient proved to be splice variants in which exon 2 was partially or completely spliced out. The deletion of exon 2 led to a complete loss of GAA activity in transfection experiments. This loss of activity is hardly surprising, since exon 2 contains three elements

essential for the maturation of GAA: (i) the translation-initiation codon, (ii) the signal peptide, and (iii) the amino-terminal proteolytic site for maturation of GAA from a 110-kD precursor to a 95-kD intermediate (Hoefsloot et al. 1990*a*; Martiniuk et al. 1991*a*; Wisselaar et al. 1993). In addition, despite the proteolytic cleavage of the amino-terminal 122 or 204 amino acids from the mature enzyme, exon 2 influences the affinity of GAA for glycogen, as shown by the diminished affinity of the GAA 2 allozyme in which there is substitution of Asn for Asp-91 in exon 2 (Martiniuk et al. 1990*a*).

The alternatively spliced nonfunctional mRNAs SV2 and SV3 were also found in the normal tissue at very low levels. It is not surprising, therefore, that they had been ignored in previous studies or that they escaped detection from the Δ18 allele. However, because exon skipping is a commonly used alternative splicing mechanism, it is possible that the nonfunctional mRNAs may be involved in the control of GAA gene expression.

In the adult patient, however, the alternatively spliced nonfunctional RNAs are the predominant products expressed from the maternal allele, and they are present at high levels relative to functional mRNA from that allele. The lesion (the −13 mutation), then, is one in which there is a profound splicing derangement leading to the up-regulation of splice variants found at very low levels in the normal tissue. The residual GAA activity observed in the patient arises from the low levels of normal mRNA transcribed from the maternal allele. The generation of low levels of normal transcript from the allele with the intron 1 mutation, described by Huie et al. (1994*b*), may be the most common mechanism for delayed phenotypic expression of the disease in 71% of GAA-deficient adults who share this mutation in heterozygous form.

It is reasonable to ask, although not yet possible to answer, what determines the age at onset in the adult patient. One possibility is that the small amount of GAA activity observed in fibroblasts taken from A40 at age 42 years has been present at that level all her life and that it was sufficient to cope with glycogen until her 5th decade, when the glycogen load increased, perhaps related to changes in metabolism with aging. Alternatively, the level of normal mRNA from the maternal allele may have fallen as the patient aged, and the phenotype may have developed only when the mRNA level reached the present level—~10% of normal. Age-related splicing variation is an established phenomenon in development and also in aging (Haverstick et al. 1990; Tanaka et al. 1992, 1993). In contrast to infants who die from Pompe disease with an overwhelming accumulation of glycogen in the liver and heart, those organs are not involved in this disease in adults, strongly suggesting that there are both tissue- and age-specific differences in regulation of the GAA gene.

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