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Characterization of Mutations in Gaucher Patients by cDNA Cloning

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Summary

Mutated cDNA clones containing the entire coding sequence of human glucocerebrosidase were isolated from libraries originated from Gaucher patients. Sequence analysis of a mutated cDNA derived from a type II Gaucher patient revealed a C-to-G transversion causing a substitution of an arginine for a proline at residue 415. This change creates a new cleavage site for the enzyme *Hha*I in the mutated cDNA. Allele-specific oligonucleotide hybridization made it possible to show that this mutation exists in the genomic DNA of the patient. From a cDNA library originated from a type I Gaucher patient, a mutated allele was cloned that contains a T-to-C transition causing a substitution of proline for leucine at residue 444 and creating a new *Nci*I site. This mutation is identical to that described by S. Tsuji and colleagues in genomic DNA from type I, type II, and type III patients. Since the new *Nci*I site generates RFLP, it was used to test the existence of this mutated allele in several Gaucher patients by Southern blot analysis. This allele was found in type I (Jewish and non-Jewish), type II, and type III Gaucher patients. These findings led us to conclude that the patient suffering from type II disease (denoted GM1260) carried both mutations described above. Any one of the amino acid changes described reduces the glucocerebrosidase activity as tested by transfection of COS cells with expression vectors harboring the mutated cDNAs. The base changes in the two mutated cDNAs do not affect the electrophoretic mobility of the corresponding polypeptides on an SDS polyacrylamide gel.

Introduction

Gaucher disease is a genetic disorder inherited as an autosomal recessive trait. It is due to the presence of an inactivated form of the lysosomal enzyme glucocerebrosidase, which in turn causes accumulation of the substrate glucocerebroside. The disease is heterogeneous and has been subdivided into three different groups: type I, the chronic adult form of the disease which does not correlate with any neurological involvement; type II, the acute type which is associated with neurological disease and produces death in early childhood; and type III, which causes a neurological disease but permits survival to adulthood (Brady et al. 1965; Frederickson and Sloan 1972; Hirschhorn and Weissmann 1976; Brady and Barranger 1983). The heterogeneity in phenotypes and the clinical variation within the different subtypes suggest the existence of several different mutations, each of which may have a different effect on the activity of the enzyme. Therefore, homozygosity for any mutated allele or combinations of two mutated alleles may result in different manifestations of the disease.

Here we report the isolation and characterization of two mutated glucocerebrosidase cDNAs. One contains a T-to-C transition substituting proline for leucine at position 444 (of the mature glucocerebrosidase), and the other contains a C-to-G transversion substituting arginine for proline at position 415. One type II patient was found to be heterozygous for both mutations.

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Material and Methods

Cells

GM1260, GM372, GM1607, GM2627, GM877,

and GM4394 are primary human skin fibroblasts from Gaucher patients (NIGMS Human Genetic Mutant Cell Repository, Camden, NJ). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplied with 20% FCS (Beth-Haemek, Israel) at 37°C with 5% CO_2. One-hundred-sixty-two are Gaucher B cells transformed with Epstein-Barr virus (EBV) (a gift from Dr. G. Lenoir, International Agency for Research on Cancer, Lyon, France). They were grown in RPMI + 20% FCS at 37°C with 5% CO_2.

RNA Preparation

A total of 2×10^7 cells were washed twice with PBS and collected in 0.2 M NaCl, 10 mM Tris pH 7.5, 1.5 mM $MgCl_2$. NP40 was added to a final concentration of 0.5%, and after 10 min at 4°C the suspension was centrifuged for 5 min at 2,000 *g* in an International centrifuge. The nuclear pellet was discarded, and an equal volume of 7 M urea, 0.3 M NaCl, 1% SDS, 10 mM EDTA Tris pH 7.5 was added to the cytoplasmic fraction. The RNA was extracted with phenol-chloroform and precipitated in ethanol. After centrifugation the RNA was fractionated through an oligo (dT) cellulose column (Aviv and Leder 1972).

cDNA Preparation

Five micrograms of poly(A) containing RNA was resuspended in 100 µl of water and treated with methyl mercury at a final concentration of 1 mM for 10 min at room temperature. One microliter of 2-mercaptoethanol was added, and the RNA was precipitated in ethanol in the presence of 0.3 M sodium acetate. The RNA was washed in 70% ethanol, and cDNA was prepared as described elsewhere (Watson and Jackson 1985).

After addition of *Eco*RI linkers and digestion with an excess of *Eco*RI, the cDNA was precipitated in ethanol and resuspended in 8 µl of 10 mM Tris pH 8.0, 1 mM EDTA. After addition of 4 µl deionized formamide and 4 µl of a dye marker, the mixture was heated to 55°C for 3 min and loaded on a 5% acrylamide gel. Electrophoresis was for 2 h at 100 V. At the end of the run the gel was stained with ethidium bromide and visualized with a UV lamp. cDNA larger than 0.5 kb was collected by electrophoresis into a dialysis bag at 70 V for 7–10 h. After passage through an Elutip-D™ column (Schleicher and Schuell) the cDNA was precipitated in ethanol.

Preparation of λgt10 Arms

λgt10 DNA was prepared as described elsewhere (Davis et al. 1980). Ten micrograms of DNA was digested with *Eco*RI (100 units) for 3 h at 37°C, followed by the addition of more enzyme (50 units) for 3 more hours. The DNA was extracted with phenol-chloroform and precipitated in ethanol in the presence of 2 M ammonium acetate.

Ligation and In Vitro Packaging

One microgram of *Eco*RI-digested λgt10 arms was added to 50 ng of cDNA in ethanol. After precipitation and washing in 70% ethanol, the mixture was resuspended in 50 mM Tris pH 8.0, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM DTT. After heating at 70°C for 3 min the mixture was incubated for 15 min at 37°C. One-half microliter of T4 DNA ligase was added (400 units of New England Biolabs ligase or 0.7 units of BRL ligase). The recombinant DNA was packaged in vitro (Gigapack; Stratagene, San Diego), and the phages were plated on the HflA150 strain of *E. coli* (Huynh et al. 1984).

Library Screening

The screening of the library was performed as described elsewhere (Benton and Davis 1977) with the normal cDNA previously cloned by us as a probe (Reiner et al. 1987).

In Vitro Transcription

In vitro transcription reactions (Melton et al. 1984) were carried out with an Amersham kit as described elsewhere (Reiner et al. 1987).

In Vitro Translation

The in vitro–transcribed RNA (Pelham and Jackson 1976) was precipitated in ethanol, centrifuged, and resuspended in 10 µl of water. Five microliters of the RNA was added to a mixture containing 8 mM phosphocreatine, 0.4 mM DTT, 20 mM Hepes pH 7.4, 70 mM potassium acetate, 0.3 mM spermidine, 20 µg creatine kinase/ml, 6 µl reticulocyte lysate, 100 µg tRNA/ml, 50 mM $MgCl_2$, and 3 µl ^{35}S-methionine (50–75 µCi; Amersham) in a total volume of 25 µl. In vitro translation was for 1 h at 37°C.

Southern Blot Analysis

Southern blot analysis was carried out as described elsewhere (Southern 1975; Reiner et al. 1988).

DNA Sequencing

Maxam and Gilbert sequencing (Maxam and Gilbert 1980) was performed on DNA fragments that were

labeled at their 3′ end with the Klenow enzyme as described elsewhere (Reiner et al. 1988). For the chain termination sequencing technique (Sanger 1981), the sequel SS/KF kit (International Biotechnologies, Inc.) was used.

5′-End Labeling of Oligonucleotides

Twenty picomoles of the 19-mer (synthesized by Dr. Ora Goldberg at the Weizmann Institute) were labeled with 40 pmol of γ-^{32}P-ATP (3,000 Ci/mmol; Amersham) by T4 polynucleotide kinase (NEN) in 100 mM Tris pH 7.5, 10 mM $MgCl_2$, 20 mM mercaptoethanol, 0.1 mM spermidine, and 0.1 mM EDTA for 1 h at 37°C. The mixture was electrophoresed through 15% acrylamide–7 M urea gel, and the labeled probe was eluted at 37°C in 10 mM Tris pH 8.0, 1 mM EDTA for 16 h.

Preparation of Gels and Hybridization to Labeled Oligonucleotides

DNA (10 μg) digested with the appropriate restriction enzyme was electrophoresed through a 0.7% agarose gel for 16 h in a Tris/borate electrophoresis buffer. The DNA was denatured in situ by being treated with 0.5 M NaOH, 0.15 M NaCl at 4°C for 30 min while being gently shaken, and was then neutralized with 0.5 M Tris pH 8.0, 0.15 M NaCl at 4°C for 30 min while being shaken gently. The gel was dried under vacuum for 1 h at room temperature and for an additional 2 hour at 60°C with a Hoefer gel dryer. The gel was then hybridized with the appropriate labeled oligonucleotide (10^9 cpm/μg) for 2 h at 57°C in 6 × SSC, 10 × Denhardt's solution, and 0.05% sodium pyrophosphate. After hybridization the gels were washed in 6 × SSC, 0.05% sodium pyrophosphate as follows: 4 × 30 min at room temperature, then 5 min at 57°C for the mutant probe and 10 min at 57°C for the normal probe. The gel was exposed to an X-ray film XAR-5; Kodak) for 4 d with an intensifying screen.

Transfection

Transfection of COS-M6 cells (Gluzman 1981; Horowitz et al. 1983) was carried out as described elsewhere (Sompayrac and Danna 1981). In essence, 10 μg of DNA in DMEM containing 0.05 M Tris pH 7.3 and 200 μg DEAE-dextran/ml (mol wt > 1 × 10^6; Pharmacia) was applied to 1–2 × 10^6 cells for 8 h at 37°C. The medium was aspirated, and, following several washes with DMEM, fresh medium containing 10% FCS was added to the cells.

Enzymatic Activity

Seventy-two hours after transfection, cells were washed twice with PBS and collected with a rubber policeman in 1 ml of PBS. The cells were precipitated, resuspended in 1 ml of sterile water, and frozen immediately at −70°C. Samples containing a known amount of protein, as determined by the Bradford technique (Bradford 1976), were tested for enzymatic activity with 4-methylumbelliferyl glucopyranoside as a substrate, as described elsewhere (Reiner et al. 1987).

Results

Characterization of a Mutated Allele from a Type II Gaucher Patient

A cDNA library in λgt10 was constructed from RNA originating from a patient with type II Gaucher disease (designated 1260). A total of 8 × 10^5 plaques from the original nonamplified library were screened using the cDNA clones previously prepared by us (Reiner et al. 1987) as probes. Five positive clones were obtained, of which two contained the entire glucocerebrosidase coding region. Their inserts were subcloned in pUC13, and the plasmids were designated p12-1-5 and p10-2-5. p12-1-5 was sequenced using the Maxam and Gilbert sequencing technique and the chain termination technique (Maxam and Gilbert 1980; Sanger 1981), as shown in figure 1*A*. A single base substitution was revealed at amino acid 415 (amino acid number 1 is the first amino acid of the mature protein, namely, alanine). A C-to-G transversion substitutes the amino acid arginine for proline (CCC to CGC, figs. 1*B*, 1*C*) and creates a new *Hha*I site (GCGC). To ensure that the mutation did not result from an error during cDNA synthesis by reverse transcriptase, the second cDNA clone (p10-2-5) was tested for the existence of the new *Hha*I site as well. The two plasmids (p12-1-5 and p10-2-5), as well as a plasmid containing the normal cDNA (p7-2-1), were digested with *Bam*HI, and the fragments were labeled at their 3′ ends with the Klenow enzyme in the presence of ^{32}P α-dGTP. A 1.6-kb *Bam*HI fragment was isolated (see map, fig. 1*A*), digested with the restriction enzyme *Hha*I, and electrophoresed through a 5% polyacrylamide gel. Since the *Hha*I site is located 180 bp upstream from the distal *Bam*HI site (see fig. 1) of the cDNA, the presence of the new *Hha*I site should be evident by the appearance of an ~180-bp fragment on the gel. The results (fig. 2) clearly demonstrate that such a band is indeed present. Thus, both cDNAs con-

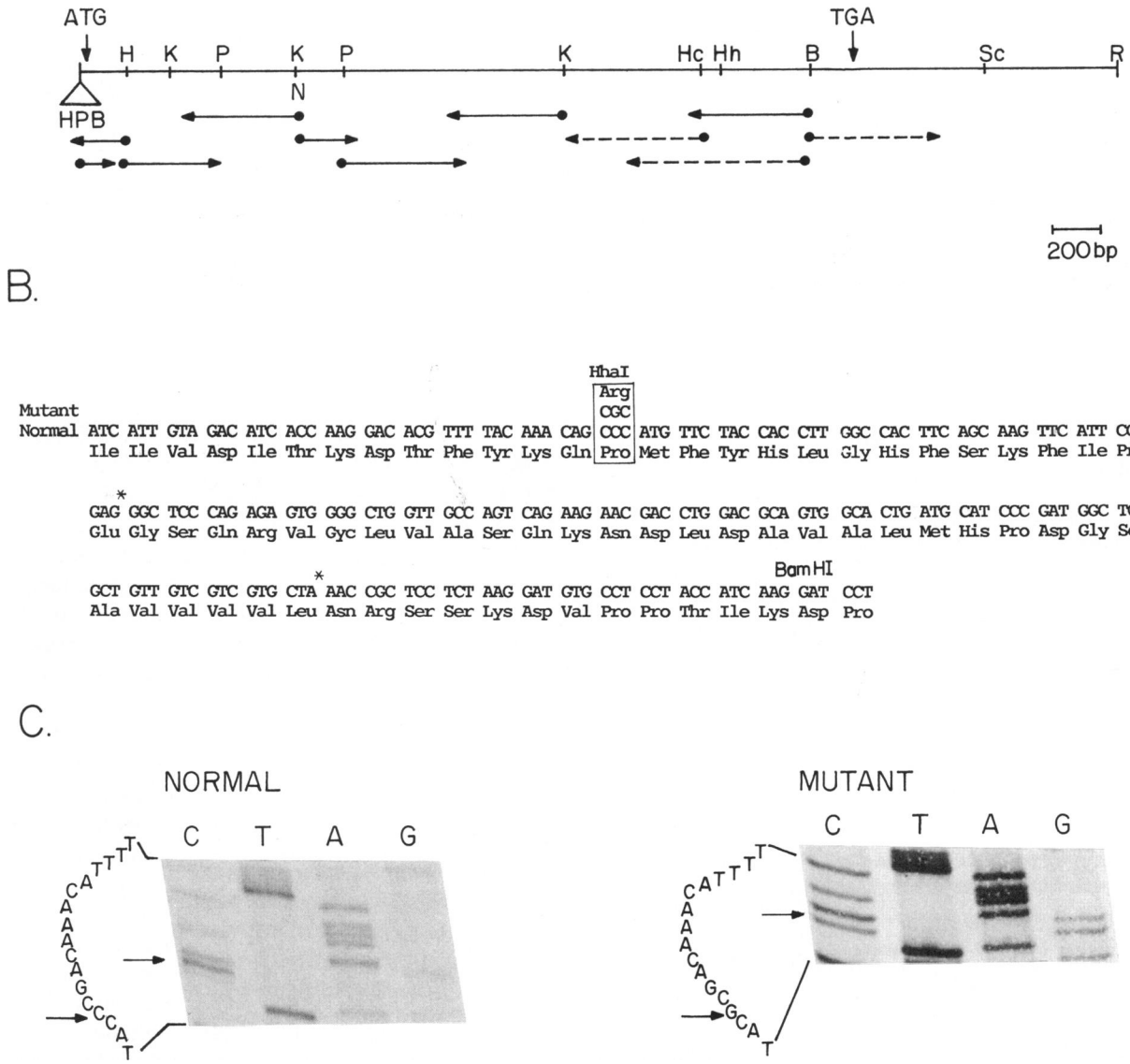

Figure 1 *A*, Strategy of sequencing the mutated allele 12-1-5 and sequence around the mutation. The arrows indicate the sequenced fragments. Hatched arrows = sequence done by the dideoxy chain termination method. Solid arrows = sequence done by the Maxam and Gilbert technique. R = *Eco*RI; H = *Hin*dIII, B = *Bam*HI; P = *Pst*I, N = *Nco*I; K = *Kpn*I; Hc = *Hinc*II; Hh = *Hha*I; Sc = *Sac*I. The positions of the glucocerebrosidase initiation and termination sites are marked by arrows. *B*, Sequence around the mutation. The stars mark the borders of the catalytic site (17). The *Bam*HI site in the sequence is the distal site of this enzyme shown on the map in panel *A*. *C*, Sequence of the normal cDNA (7-2-1) and the mutated allele (12-1-5) showing the C-to-G transversion.

tain a new *Hha*I site that does not appear in the normal cDNA, which suggests that the mutation found is authentic and does not represent a cDNA cloning error.

To prove unequivocally that the mutation is indeed present in the gene, allele-specific oligonucleotide hybridization was performed. To that end, genomic DNA was prepared from either normal embryonic tissue or tissue from patient 1260, from which the mutated cDNA

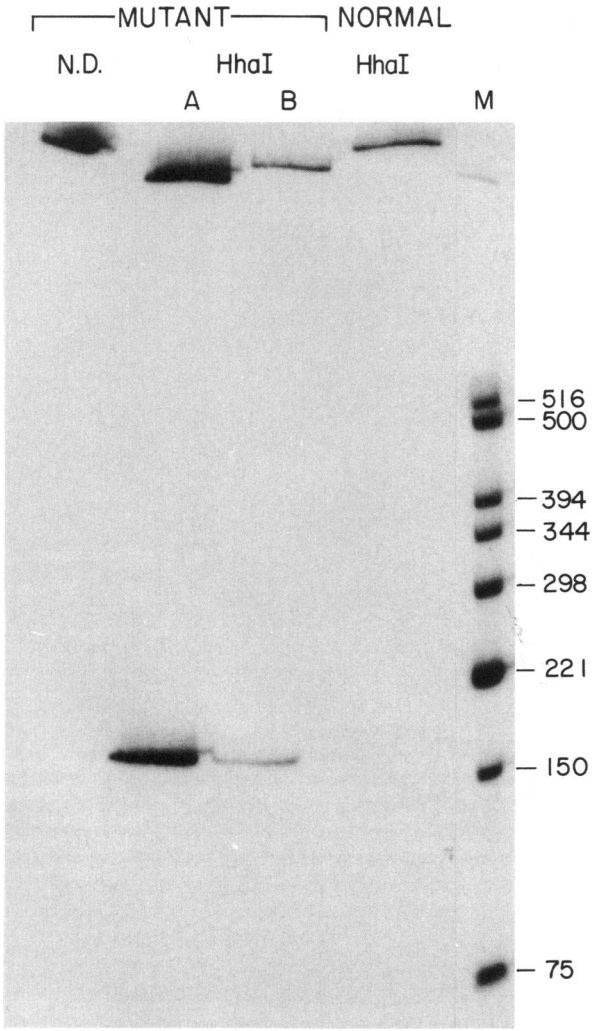

Figure 2 Digestion of the mutated cDNA clones with *Hha*I. Two different mutated glucocerebrosidase cDNA inserts isolated from a nonamplified 1260 cDNA library as well as a normal glucocerebrosidase cDNA clone were digested with the restriction enzyme *Bam*HI for 1 h at 37°C. One microliter of ^{32}P-α-dGTP was added with 5 units of the *E. coli* DNA polymerase I Klenow fragment for 1 h at 37°C. At the end of the incubation, the DNA was electrophoresed through a 5% polyacrylamide gel, and a 1.6-kb fragment (see fig. 1 for map) was electroeluted and precipitated in ethanol. The fragment was resuspended in 30 μl of 50 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT and was digested with the restriction enzyme *Hha*I, after which it was electrophoresed through a 5% polyacrylamide gel and autoradiographed. N.D. = nondigested (12-1-5) DNA; A = 12-1-5 DNA; B = 10-2-4 DNA; NORMAL = normal cDNA (7-2-1); M = markers.

clones were derived. Ten micrograms of DNA were digested with *Bam*HI and electrophoresed through an agarose gel. The gel was dried and hybridized to synthetic oligonucleotides bearing either the normal or the mutated sequence. The results (fig. 3) demonstrate that the "normal" oligonucleotide hybridized to two bands representing the two genes. There are two human glucocerebrosidase genes—one is an active gene designated 6-1 and the other, which is a pseudogene, has been designated 10-2 (Reiner et al. 1988). Since they differ in size, their cleavage with *Bam*HI and hybridization with the oligonucleotide yield different size fragments. The "mutated" oligonucleotide hybridized only to the mutated gene present in patient 1260 but not in the normal embryonic DNA.

Isolation of a Mutated Allele from a Type I Gaucher Patient

A cDNA library in λgt10 originating from a type I (GM4394) Gaucher patient was constructed and screened. Several phages containing the entire glucocerebrosidase coding sequence were obtained, and one designated p8-1-1 was analyzed, its insert being sequenced by means of the chain termination reaction (fig. 4*A*). As shown in figure 4*B*, one base change was found at a position corresponding to amino acid 444. This transition (T to C) causes substitution of leucine for proline (CTG to CCG) and creates a new *Nci*I site. Two silent changes were found as well: ATT to ATC at amino acid 260 and GTG to GTC at residue 431.

Southern Blot Analysis of Mutated DNAs

Both mutations described above create new restriction sites, for *Hha*I and *Nci*I, which should be very useful for identifying these mutated alleles in different Gaucher patients. However, the sequence GCGC, which constitutes the *Hha*I recognition site, appears to be highly methylated in genomic DNAs. Since *Hha*I does not recognize methylated sequences, the mutation which creates a new *Hha*I site at residue 415 cannot be diagnosed simply by following RFLP. On the other hand, the transition from a T to a C at residue 444 creates a new *Nci*I site, which can be digested. Several genomic DNAs from Gaucher patients were digested for 48 h with *Nci*I. The samples were electrophoresed through a 0.7% agarose gel, blotted, and hybridized with an appropriate probe (see fig. 5*A*, 5*B*). As shown in fig. 4*C*, the normal active glucocerebrosidase gene (6-1) should yield a 2.6-kb *Nci*I fragment when hybridized to the probe. The other gene, (10-2), which is a pseudogene (Horowitz et al., in press), should yield two fragments of 1.4 and 1.0 kb, since it normally contains an *Nci*I site at a position corresponding to residue 444 (M. Horowitz, unpublished data). However, if the normal gene (6-1) is mutated at residue 444 such that there is

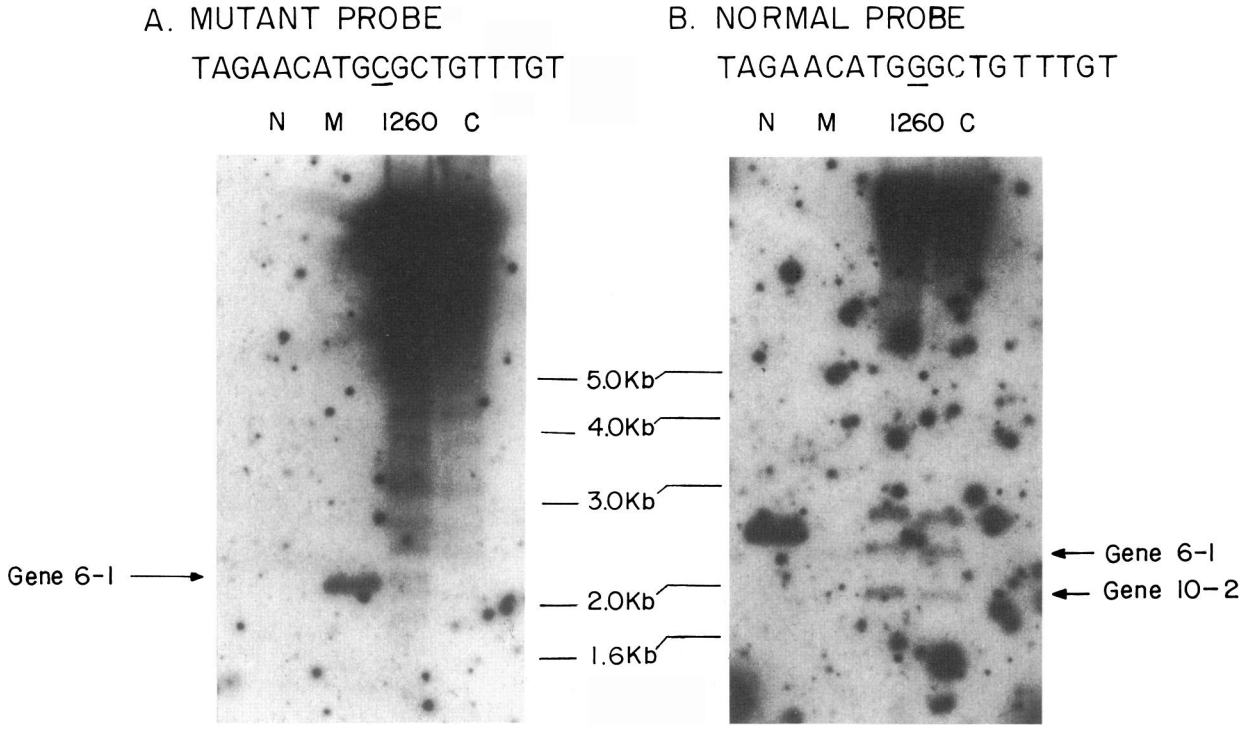

Figure 3 Hybridization of oligonucleotide probes to gels containing genomic DNA. Ten micrograms of human embryonic DNA or DNA extracted from skin fibroblasts of patient 1260 was digested with *Bam*HI for 16 h at 37°C and electrophoresed through a 0.7% agarose gel for 16 h. *Eco*RI digests of 100 pg p7-2-1 (normal cDNA in pUC) and of p12-1-5 (the mutated cDNA in pUC) were also included in this gel. The gel was processed and hybridized to the end-labeled oligonucleotides as described in Material and Methods. *A*, Hybridization to the mutated probe. *B*, Hybridization to the normal probe. The arrows indicate the fragment deriving from the two human glucocerebrosidase genes. C = control embryonic DNA; 1260 = DNA from patient 1260; M = p12-1-5; N = p7-2-1.

a new *Nci*I site, two fragments, one 1.6 kb and one 1.0 kb in size, are expected (fig. 5*C*). Therefore, the appearance of a 1.6-kb fragment is diagnostic of the existence of the "*Nci*I" mutation in the active glucocerebrosidase gene. As shown in figure 5*A*, the 1.6-kb fragment indeed appears in the patients designated GM1260 and GM877 (type II), GM372 (type III), GM1607 (type I, Jewish), and GM4394 and 162 (type I, non-Jewish). As described above, GM1260 contains also the other mutation (the "*Hha*I" mutation). Thus, GM1260 is a compound heterozygote. Table 1 summarizes the results concerning the existence of the *Hha*I or the *Nci*I mutations in different patients.

Transient Expression of the Mutated cDNAs in COS-M6 Cells

To prove that the amino acid substitutions described above alter the enzymatic activity of the corresponding proteins, the mutated cDNAs were coupled to the SV40 late promoter in an SV40 shuttle vector (pSVL; Pharmacia). The vectors were used for transfection of COS-M6 cells. Seventy-two hours after transfection, cell lysates were prepared and glucocerebrosidase activity was assayed with 4-methylumbelliferyl glucopyranoside as a substrate. As shown in figure 6, in COS-M6 cells transfected with the normal cDNA, glucocerebrosidase activity increased 180% compared with nontransfected cells, while in cells transfected with the mutated cDNAs the glucocerebrosidase activity was below that for the nontransfected control cells.

In Vitro Transcription-Translation of the Mutated cDNAs

Recently, the catalytic site of the human glucocerebrosidase has been mapped with the use of the inhibitor conduritol β-epoxide, which is known to bind covalently to acidic amino acids in the catalytic sites of glucosidases (Dinur et al. 1986). The catalytic site spans the region encompassed by amino acids 430 and 461. Therefore, the mutation at position 415 (the "*Hha*I" mutation) maps upstream from the catalytic site, though

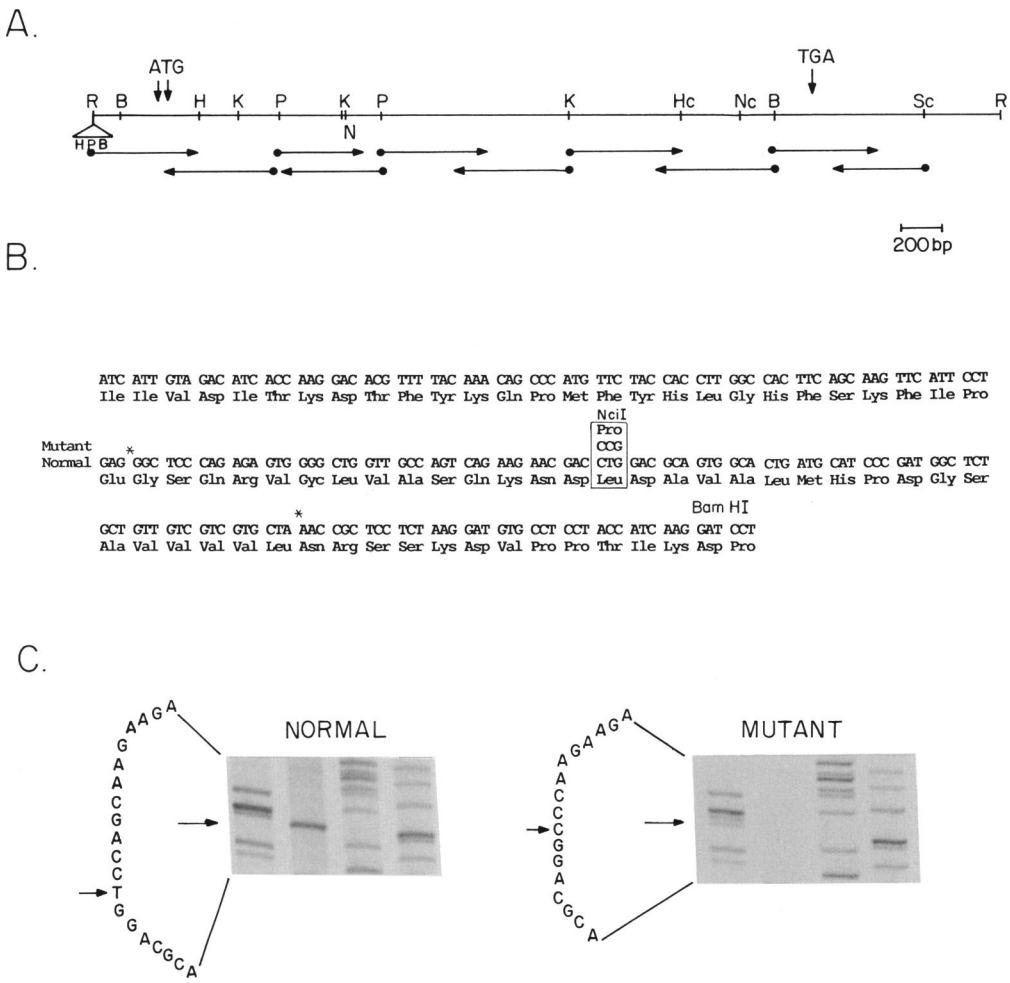

Figure 4 Strategy of sequencing the mutated allele designated 8-1-1. *A*, The arrows indicate the sequenced fragments. Sequencing was performed by the dideoxy chain termination technique. R = *Eco*RI; H = *Hin*dIII; B = *Bam*HI; P = *Pst*I; N = *Nco*I; Nc = *Nci*I; K = *Kpn*I; Hc = *Hin*cII; Sc = *Sac*I. *B*, Sequence around the mutation in the allele 8-1-1. The stars mark the borders of the catalytic site (*A*). The *Bam*HI site in the sequence is the distal site of the enzyme shown in 4*A*. *C*, Sequence of the normal cDNA (7-2-1) and the mutated allele (8-1-1) showing the T-to-C transition.

in its close vicinity, while the mutation at position 444 (the "*Nci*I" mutation) maps within the catalytic site. These two mutations, which substitute arginine for proline and proline for leucine, respectively, may change the tertiary structure of the protein and therefore its catalytic activity. Changes involving proline residues have been shown to affect the mobility of the protein on polyacrylamide-SDS gels (Harris et al. 1987; Matlashewski et al. 1987). It was therefore interesting to test whether the electrophoretic mobilities of the primary products of the mutated glucocerebrosidase alleles are also changed. To do so, the mutated cDNAs (12-1-5 and 8-1-1), as well as the normal counterparts (12-1-5/N and 7-2-1), were coupled to the SP6 polymerase promoter in the vector Gemini-2 (Promega; USA) and RNA was transcribed from these plasmids. The RNAs were translated in vitro in the reticulocyte cell–free lysate system (Pelham and Jackson 1976), and the products were electrophoresed through a 10% polyacrylamide-SDS gel (Laemmli 1970). As shown in fig. 7, the mobility of the nascent mutated polypeptides was indistinguishable from that of the normal counterparts.

It is worth mentioning that the glucocerebrosidase mRNA has two in-frame ATGs, which may be used in vivo for translation initiation, although the first ATG is not preceded by a typical (Kozak) consensus sequence (Fig. 7*B*) (Sorge et al. 1985; Kozak 1986). The two

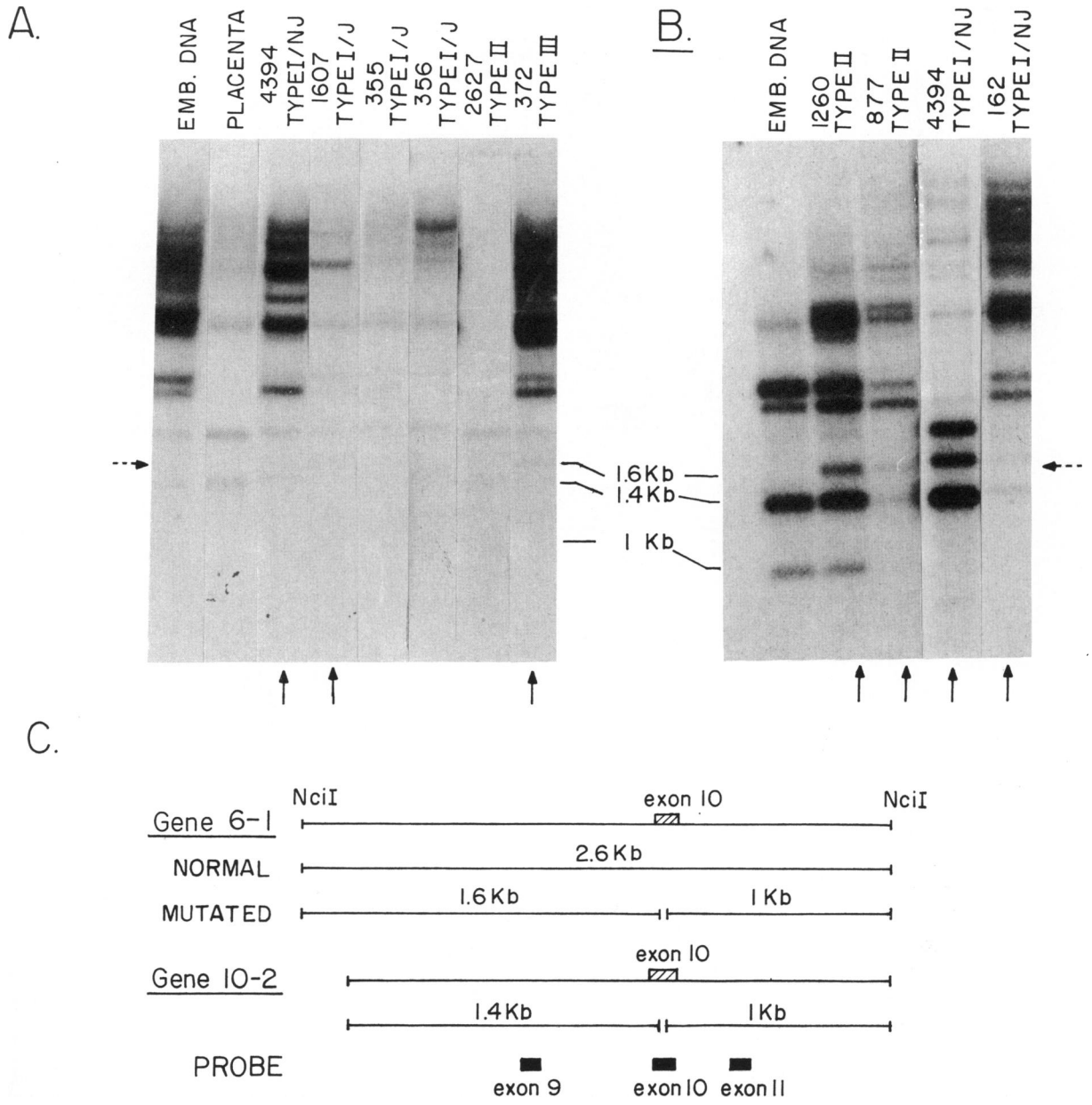

Figure 5 Southern blot analysis of genomic DNAs. Ten micrograms of normal placental DNA or DNA extracted from an embryo (EMB. DNA) or DNA extracted from different Gaucher cells was digested with the restriction enzyme *Nci*I for 48 h at 37°C. The digests were electrophoresed through a 0.7% agarose gel for 20 h at 30 V. Following denaturation and neutralization the gel was blotted onto nitrocellulose paper (Hybond C™; Amersham) for 18 h. The filter was baked for 2 h at 80°C, prehybridized for 2 h at 42°C in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 50 mM sodium phosphate pH 6.8, and 50 μg denatured salmon sperm DNA/ml, and hybridized for 48 h at 42°C with a ^{32}P-labeled probe prepared from an appropriate M13 clone (14). *A*, Hybridization with a 180-bp *Bam*HI-*Hha*I fragment derived from the allele 12-1-5 (see fig. 1). NJ = non-Jewish; J = Jewish. *B*, Hybridization with a 450-bp *BamHI-Kpn*I fragment derived from the normal glucocerebrosidase cDNA. *C*, Map of the *Nci*I fragments expected from the two glucocerebrosidase genes (designated 6-1 and 10-2) and the mutated counterpart of the gene 6-1. The arrow denotes the 1.6-kb fragment, specifying the *Nci*I mutation.

Table 1

Summary of Mutations Found in Different Patients

Patient	Type of Disease	Mutation(s)	Source of Sample, Reference
GM1260	II	*NciI/HhaI*	Skin fibroblast, cell repository, New Jersey
GM4394	I(NJ)	*NciI*	Skin fibroblast, cell repository, New Jersey
GM372	III	*NciI*	Skin fibroblast, cell repository, New Jersey
GM2627	II	...	Skin fibroblast, cell repository, New Jersey
GM877	II	*NciI/NciI*	Skin fibroblast, cell repository, New Jersey
GM1607	I(J)	*NciI*	Skin fibroblast, cell repository, New Jersey
355	I(J)	...	Blood, Tel-Hasomer, Israel
356	I(J)	...	Blood, Tel-Hasomer, Israel
162	I(NJ)	*NciI*	EBV-transformed B cells, G. Lenoir

NOTE.—NJ = non-Jewish; J = Jewish.

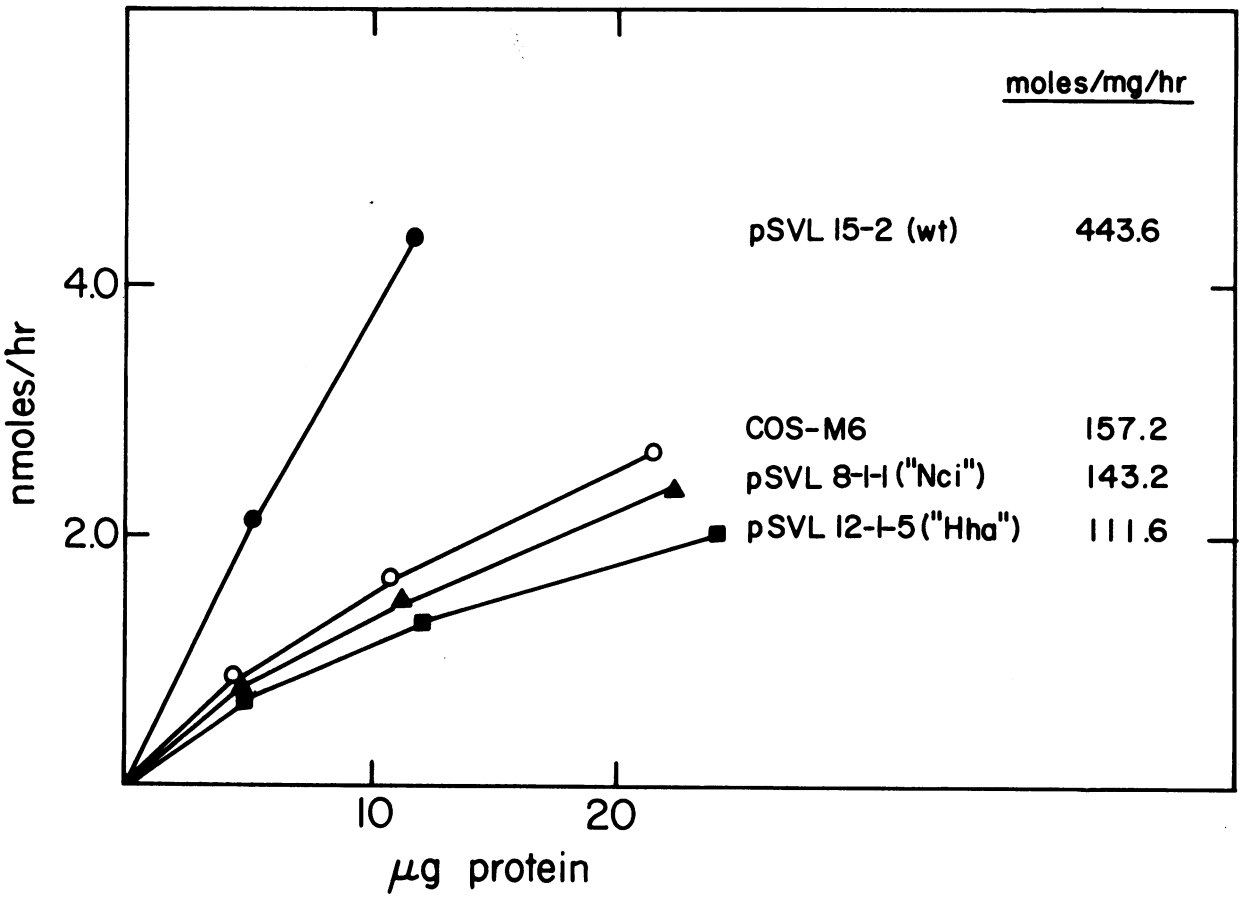

Figure 6 Expression of the mutated cDNAs in COS-M6 cells. The mutated cDNAs (12-1-5 and 8-1-1) or the normal cDNA (15-2, a cDNA recently cloned and sequenced by us) were coupled to the SV40 late promoter in the pSVL vector (Pharmacia) at the *SmaI* site. Ten micrograms of the different pSVL-derived vectors was used to transfect COS-M6 cells by the DEAE-dextran method as described in Material and Methods. Seventy-two hours later, cell lysates were prepared and glucocerebrosidase activity was assayed as described in Material and Methods.

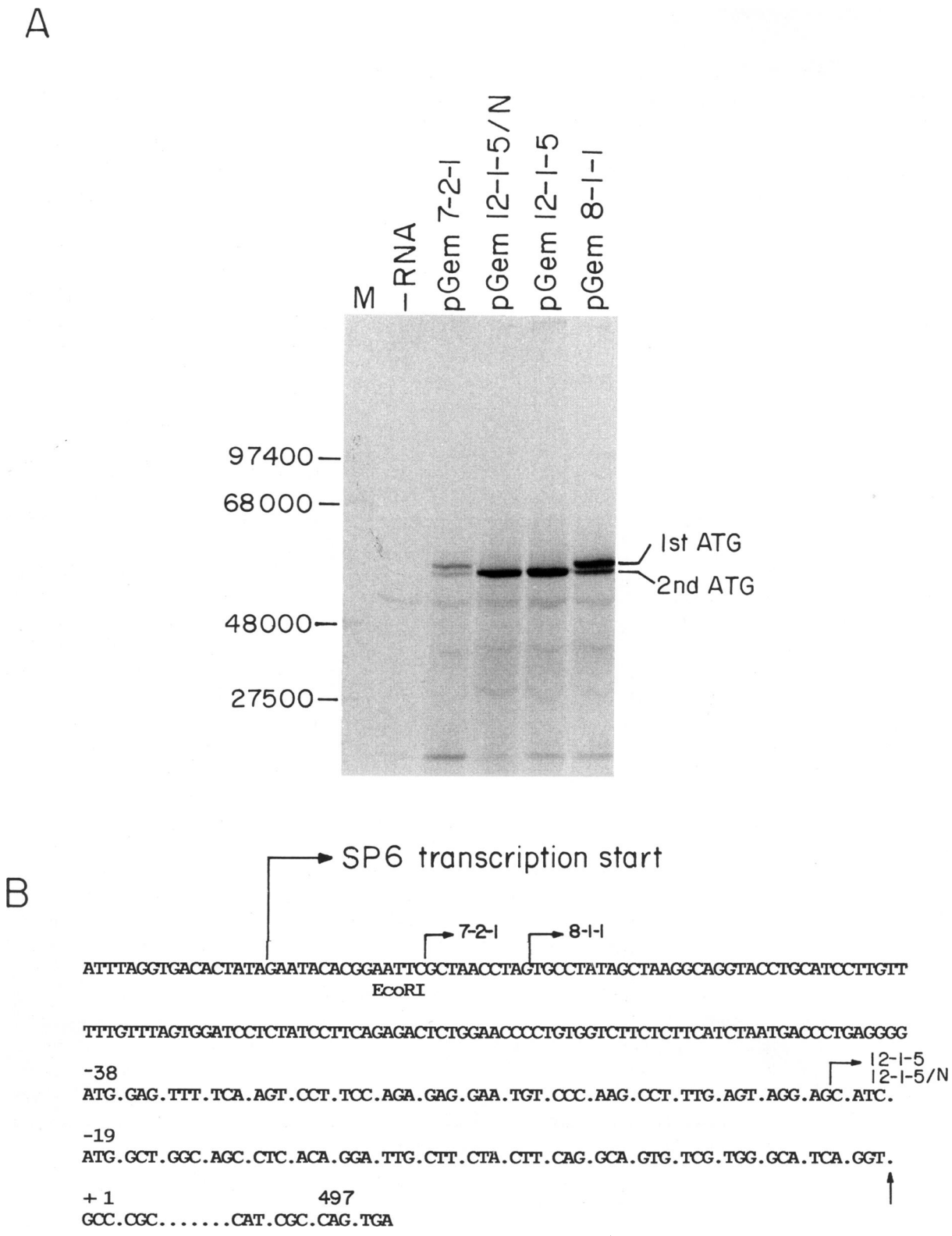

Figure 7 SDS-PAGE of in vitro translated proteins. *A*, Five micrograms of Gemini-2 derived plasmids containing normal or mutated glucocerebrosidase cDNAs were linearized with the restriction enzyme *Sma*I, extracted with phenol-chloroform, and ethanol precipitated. The DNAs were resuspended in 5 μl water and transcribed in vitro according to the Amersham protocol for 1 h at 37°C. After precipitation in ethanol the RNA was resuspended in 10 μl of water, and 5 μl was added to an in vitro translation mix, as explained in Material and Methods. After an incubation of 1 h at 37°C, 10 μl of dye was added. The mixture was boiled for 3 min and electrophoresed through a 10% polyacrylamide-SDS gel for 3 h at 120 V. The gel was stained, destained, dried, and exposed to an X-ray film (Curix, Agfa) for

cDNAs mentioned above (12-1-5 and 12-1-5/N) have only the second ATG, which was used efficiently, as can be anticipated from figure 7. However, when longer cDNA clones (7-2-1 and 8-1-1) containing both ATGs were used for the in vitro transcription-translation reactions, both initiation codons were efficiently utilized by the translation machinery.

Discussion

We report here the finding of two mutated cDNAs derived from Gaucher patients. One allele isolated from a type II Gaucher patient (designated 1260) contains a C-to-G transversion at codon 415, which causes substitution of arginine for proline and creates a new *Hha*I restriction site. The other mutation is a T-to-C transition at codon 444, a transition that causes a substitution of proline for leucine and creates a new *Nci*I restriction site. This mutated allele was isolated from a type I non-Jewish patient (GM4394). The *Nci*I mutation has been reported recently by Tsuji et al. (1987), who cloned a mutated gene from a patient. They found the "*Nci*I" mutation in either a homozygous or a heterozygous state in type II and type III patients and in a heterozygous state in type I patients, and suggested that it may serve as a marker for patients suffering from the neurological disease.

The creation, by mutations, of new restriction sites—and, therefore of new restriction fragments—enables easy diagnosis of such mutations in genomic DNA samples. It turns out that total human DNAs are digested fairly well with *Nci*I, but it is practically impossible to digest them satisfactorily with *Hha*I. The latter difficulty probably reflects the fact that the sequence GCGC, which is the recognition signal for *Hha*I, is highly methylated in genomic DNA and therefore cannot be cleaved by this methylation-sensitive enzyme. Therefore, the new restriction site for *Hha*I cannot be easily used for diagnostic purposes. However this mutation can be diagnosed easily in genomic DNA by a combination of the allele-specific oligonucleotide hybridization and in vitro amplification of genomic sequences by using the polymerase chain reaction (Saiki et al. 1985; Mullis and Faloona 1987). Using the *Nci*I site as a diagnostic marker, we discovered several Gaucher patients that have this mutation, and they include type I non-Jewish (162 and GM4394), type I Jewish (GM1607), type II (GM1260 and GM877), and Type III (GM372) patients. Moreover, with a combination of the polymerase chain reaction DNA amplification technique and the allele-specific oligonucleotide hybridization we are able to discriminate between homozygosity and heterozygosity for the *Nci*I mutation (data not shown). We found that the patients designated 1607, 1260, 372, and 4394 are heterozygotes, while patient 877 is homozygous for the *Nci*I mutation. The mutations described above map within the active site (the *Nci*I mutation) or close to it (the *Hha*I mutation). Since the substitutions exchange proline for leucine and arginine for proline, they may change the tertiary structure of the polypeptide and therefore its catalytic activity.

The amino acid substitutions resulted in proteins with nondetectable enzymatic activity. The human glucocerebrosidase synthesized in COS-M6 cells transfected with the mutated cDNAs coupled to the SV40 late promoter was inactive compared with the enzyme directed by the normal cDNA coupled to the SV40 late promoter in the same shuttle vector. Tsuji et al. (1988) showed that, in COS cells transfected with another human mutant glucocerebrosidase cDNA coupled to the SV40 early promoter, there was no significant increase in glucocerebrosidase activity, while, in cells transfected with the normal cDNA clone, there was a 155% increase in the glucocerebrosidase activity. The electrophoretic mobilities of the mutated nascent polypeptides were not changed in comparison with those of their normal counterpart. In the human p53 proteins which are encoded by the same gene (Harris et al. 1987; Matlashewski et al. 1987) a similar base change from G to C substitutes arginine for proline. However, in this case the two proteins have different mobilities on a polyacrylamide-SDS gel.

The glucocerebrosidase mRNA contains two ATGs in frame which are 19 amino acids apart (Sorge et al. 1985). It is interesting to note that both of them are efficiently translated in vitro (see fig. 7 and Sorge et al. 1987), although the first one is not preceded by a typical consensus sequence (Kozak 1986). The same ob-

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24 h. 8-1-1 = The mutated cDNA containing the "*Nci*I" mutation; 12-1-5 = the mutated cDNA containing the "*Hha*I" mutation; 7-2-1 = the normal cDNA that contains two ATGs in frame (14, 19); 12-1-5/N = the normal cDNA that contains exactly the same 5′ end as 12-1-5. *B*, Illustration of the sequence of the different SP6-derived vectors, depicting the SP6 transcription start, the 5′ ends of the different cDNA clones (7-2-1, 8-1-1, 12-1-5, 12-1-5/N), the two ATGs (the ATG at position −38 is the first one in frame, the second one is at position −19), and the termination codon TGA. The arrow denotes the cleavage site of the leader. The first amino acid downstream from this cleavage site is residue 1.

servation has been made for the human chronic granulomatous disease (CGD) gene (Dinauer et al. 1987). The CGD cDNA isolated contains two potential initiator codons. On the basis of consensus rules for selection of functional ATGs (Kozak 1986), only the second ATG is compatible. However, both ATGs were used in in vitro translation systems.

In addition to the mutations described in the present work, several other single-base differences have been found between independent isolates of glucocerebrosidase cDNAs (Sorge et al. 1985; Tsuji et al. 1986). These differences include: GGA instead of GGT at residue 13 (no amino acid change), GCC instead of GCA at residue 164 (no amino acid change), CCT to CTT at residue 470 (changing proline to leucine), and CAT to CGT at residue 495 (changing histidine to arginine). These changes are most probably due to polymorphism in the normal human glucocerebrosidase gene, since both clones were derived from non-Gaucher cells. In addition, both published cDNAs were biologically active on transfection of different cells with expression vectors harboring them (Choudary et al. 1986; Sorge et al. 1986).

The finding of several mutations in Gaucher patients located in different domains of the enzyme may enable us to explain the phenotypic heterogeneity found in this disease. Moreover, it may serve as a useful tool for diagnosis and accurate typing of the disease.

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