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ORIGINAL INVESTIGATION

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# Molecular analysis of the ß-glucuronidase gene: novel mutations in mucopolysaccharidosis type VII and heterogeneity of the polyadenylation region

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**Abstract** We used polymerase chain reaction (PCR)/single-strand conformation polymorphism analysis and direct sequencing of the coding region of the β-glucuronidase cDNA and gene to detect mutations causing β-glucuronidase enzyme deficiency in five MPS VII patients. Four patients presented with hydrops fetalis, one with an early infantile form of the disease. Genetic heterogeneity of MPS VII alleles was further confirmed in this study. Recurrent mutations were observed in patients of related origin. Previously unknown alleles detected were R110X, F361Δ9, 1270 + 1G→A, S52F and 1480Δ4. Reverse transcription/PCR analysis of the 1270 + 1G→A messenger showed aberrant splicing: inclusion of intron 7 or skipping of exons 6–7 and 9. Messenger RNA transcribed from the R110X and 1480Δ4 alleles was unstable. We detected a 2154A/G change in the 3′ non-coding region of the gene, in the neighbourhood of the two consensus polyadenylation sites. 3′-Rapid amplification of cDNA ends/PCR of fibroblast cDNA revealed equal usage of two alternative polyadenylation sites. The 2154A/G substitution did not influence adenylation-site choice, nor the amount of stable messenger produced. The finding that 2 out of 30 normal controls carried the 2154G allele indicated that the 2154A/G substitution is a harmless polymorphism. The S52F and F361Δ9 cDNAs were constructed in vitro and used to transfect COS cells transiently. Both mutations completely abolished enzyme activity.

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## Introduction

Deficiency of the lysosomal enzyme β-glucuronidase (E.C.3.2.1.31) causes mucopolysaccharidosis type VII (MPS VII; Sly et al. 1973; reviewed by Neufeld and Muenzer 1995). An extremely wide spectrum of clinical presentations of the disease has been observed. Diagnosis of the disease therefore greatly depends on laboratory findings, such as low β-glucuronidase enzyme activity, mucopolysaccharidosis and the presence of granulations in many tissues. The existence of a pseudodeficiency allele for the β-glucuronidase gene further complicates biochemical diagnosis of MPS VII (Chabas et al. 1991; Vervoort et al. 1995).

The cDNA and the gene coding for human β-glucuronidase have been cloned and characterized (Oshima et al. 1987; Miller et al. 1990). Multiple unprocessed pseudogenes complicate the molecular analysis of the gene. Methods for selective amplification of the gene by the polymerase chain reaction (PCR) have been designed (Shipley et al. 1993; Vervoort et al. 1993, 1996), allowing genetic studies of MPS VII patients; these studies have shown extensive genetic heterogeneity (Tomatsu et al. 1990, 1991; Shipley et al. 1993; Vervoort et al. 1993, 1995, 1996; Wu and Sly 1993; Wu et al. 1994; Yamada et al. 1995). Genetic heterogeneity has also been observed in a relatively homogeneous clinical phenotype (i.e. hydrops fetalis). In the present study, we have analysed the β-glucuronidase gene and cDNA of five MPS VII patients. To investigate the effect of a 2154A/G change on polyadenylation and mRNA stability, we have used 3′-rapid amplification of cDNA ends (RACE)/PCR analysis of the β-glucuronidase cDNA.

## Subjects, materials and methods

### Subjects

Patient 1 was born to a non-consanguineous couple from the Netherlands. The index case was a 16-week hydropic fetus. Cultured amniocytes were available for study. Two previous pregnancies resulted in an affected hydropic fetus. In the first pregnancy, which ended after 34 weeks, hydrops was detected at week 25; increased intra- and extra-medullary haematopoiesis were documented. In the second pregnancy, hydrops, granulocytopenia and anaemia were noted at week 15. One healthy child was born to this couple.

Patient 2 was of Mexican descent. The parents denied consanguinity. At 17 weeks, mild oedema was observed on ultrasound; the fetus developed ascites, pleural effusions and an echogenic bowel. Analysis of fetal umbilical blood lymphocytes by electron microscopy showed numerous lysosomes with a particulate content. The pregnancy was terminated at 23 weeks. Total RNA and genomic DNA were prepared from frozen tissue (heart, liver). One previous pregnancy ended with a 30-week fetal demise. The oedematous baby was otherwise anatomically normal.

Patient 3 was a severely affected child from Czechoslovakia. Symptoms were present at birth: hepatosplenomegaly, pectus carinatum and infundibular formation of the thorax. At 5 months of age, bilateral inguineal hernia, slight hypertelorism, hydrocoele and hypergranulation of leucocytes were observed. The infant had psychomotoric retardation and macrocephaly. Cultured fibroblasts were available for this study.

Patient 4, from Belgium, was described as a hydropic fetus (Lissens et al. 1991). Detection of the paternal mutation of this fetus has been reported previously (Vervoort et al. 1993).

Patient 5 was a hydropic fetus of Belgian origin. Hydrops fetalis was noted on ultrasound in two previous pregnancies. In the first pregnancy, rapidly increasing ascites, subcutaneous oedema and placental thickening were noted at 22 weeks. Echocardiography showed no structural cardiac defects or arrhythmia. Microscopy of the placenta showed prominently vacuolated Hofbauer cells. Histology of the fetus showed numerous vacuolated macrophages in liver, spleen, lymph nodes and bone marrow. Scattered foamy macrophages were seen in the lungs, the myocardium, the adrenals and the kidneys. Renal tubular cells were vacuolated but stained only faintly for mucopolysaccharides. Microscopically, the heart showed a slightly thickened fibrotic endocardium. In the second pregnancy, oedema of the neck and the back was noted at 12 weeks' gestation (this neck oedema had also been seen at an early stage in the first pregnancy). As in the previous fetus, no other macroscopic abnormalities were found. The third fetus was also hydropic and, at autopsy, no other abnormalities were found. Cultured amniocytes of the third pregnancy were used for mutation analysis.

### RNA preparation, reverse transcription/PCR, single-strand conformation polymorphism analysis and sequencing

Isolation of total RNA, reverse transcription (RT)/PCR, single-strand conformation polymorphism analysis (SSCP) and sequencing of PCR products were performed as reported previously (Vervoort et al. 1996). The oligonucleotides used here for PCR and sequencing were as defined in Shipley et al. (1993) and Vervoort et al. (1996).

### Genomic DNA preparation, PCR, SSCP and sequencing

Genomic DNA was prepared from the material available. Amplification with PCR, SSCP and sequencing of PCR products were performed as reported previously (Vervoort et al. 1996). To amplify the promoter region and exon 1 of the β-glucuronidase gene, we used primer pair RAF26-*1r* as described; a second PCR amplification of this product with hemi-nesting primer pairs RAF26-RAF25 and RAF1-*1r* was employed. These PCR products were better templates for direct sequencing. Exon 9 sequences were determined in the sense direction by RAF13, with a cycle sequencing protocol (Thermo-Sequenase, Amersham).

### 3′-Rapid amplification of cDNA ends/PCR

For 3′-rapid amplification of cDNA ends (RACE), we used a kit for RT-PCR from Perkin Elmer. However, RT was primed with the Not *I*-$d(T)_{18}$ primer supplied by Pharmacia. The first PCR amplification was carried out with sense primer RAF13 and the Not27 antisense primer (consisting of the anchor portion of the Not *I*-$d(T)_{18}$ primer). This product was reamplified with primer pair RAF15-Not27 and the subsequent product was purified on agarose gel, reamplified and sequenced with primers RAF15 and HUG16; sequencing of the 3′ end was carried out with primer RAF48 (cDNA position 1981–2000, 5′-GAGCAAGACTGATACCACCT-3′), by using Thermo-Sequenase (Amersham).

### Expression studies

For expression in COS cells, mutations were introduced into the wild-type β-glucuronidase cDNA cloned in the expression vector pJC119 (Oshima et al. 1987). We amplified cDNA with PCR, derived mutant restriction fragments and replaced the fragment in the expression vector. Mutant constructs were verified by sequencing the appropriate region of the cDNA.

## Results

### Detection of MPS VII mutations

Methods for mutation analysis of the β-glucuronidase cDNA and gene have been described previously (Shipley et al. 1993; Vervoort et al. 1996). The deleterious alterations found on MPS VII alleles of patients 1–5 are summarized in Table 1 and Fig. 1. The 1107Δ27 deletion was easily detected when PCR products were run on agarose or polyacrylamide gels and were localized in a 55-nt interval (nt 1092–1146). The second allele of patients 1 and 5 resulted in greatly reduced amounts of stable mRNA (RNA-). Restriction analysis with *Bgl*II of PCR-amplified exons 6–7 from genomic DNA of patient 1 revealed an R357X allele. In order to find the MPS VII mutation on the second chromosome of patient 5, PCR fragments amplified from genomic DNA were sequenced; an 1480Δ4 deletion of 4 nt in exon 9 was the only other change found. Aberrant PCR products amplified from the cDNA of patient 2 suggested a splice-site mutation. SSCP analysis of PCR-amplified exons 6–7 and exons 7–8 from genomic DNA of this patient showed altered mobility of both fragments. Direct sequencing disclosed the 1270 + 1G→A transition in the first nucleotide of intron 7, localized in the overlap of the fragments analysed with SSCP. As this change creates a new restriction site for *Nla*III, it could be confirmed unambiguously after digestion of PCR products with this enzyme. The R374C change (patient 3) was detected with PCR-SSCP and direct sequencing of the cDNA. Although the second allele of this patient was equally expressed in his mRNA, no other

**Table 1** Summary of MPS VII mutation analysis

| Patient number and origin | Phenotype | Nucleotide change[a] | Amino acid change[a] | Exon | Effect on RNA[a] | Haplotype[c] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | 1766 | 1972 | 2154 |
| Patient 1 | Hydrops | 1107Δ27 | F361Δ9 | 7 | – | C | C | n.d. |
| The Netherlands | | 1095C→T | R357X | 7 | RNA- | C | C | n.d. |
| Patient 2 | Hydrops | 1270 + 1G→A[b] | – | Intron 7 | Aberrant | C | C | n.d. |
| Mexican descent | | 1270 + 1G→A | – | Intron 7 | splicing | C | C | n.d. |
| Patient 3 | Neonatal | 1146C→T | R347C | 7 | – | C | T | G[d] |
| Czechoslovakia | | 181C→T | S52F | 1 | – | C | C | A |
| Patient 4 | Hydrops | 672C→T | R216W | 4 | – | C | T | A |
| Belgium | | 354C→T | R110X | 2 | RNA- | C | T | A |
| Patient 5 | Hypdrops | 1107Δ27 | F361Δ9 | 7 | – | C | C | A |
| Belgium | | 1480Δ4 | frameshift | 9 | RNA- | C | C | A |

[a] Both alleles of each patient are given. – No effect

[b] Unable to exclude a full deletion of the β-glucuronidase gene on a patient's second chromosome

[c] Four polymorphic loci were analyzed: 1091 + 27C/G, 1766T/C, 1972C/T and 2154A/G. The 1091 + 27C or G allele was always associated with the same 1972T or C allele, respectively; therefore, analysis of 1091 + 27C/G is not listed. *n.d.* Not determined

[d] Three changes were found on both alleles of patient 3 (S52F, R374C, 2154G). The 2154A/G change is probably a (relative rare) harmless polymorphism. 2154G was assigned to his R374C allele, since it was also found on the R374C allele of another MPS VII patient. As a consequence, the only change on the second allele of patient 3 was the S52F missense mutation, which destroys enzyme activity completely in a transfection experiment

changes were disclosed with RT-PCR-SSCP. After complete sequencing of the coding region of both the cDNA and the gene, we found that this patient also carried an S52F allele. Molecular analysis of the cDNA of the fourth patient showed that he was a compound heterozygote for a R216W allele and a second allele not stably expressed in his mRNA (Vervoort et al. 1993). After complete sequencing of the coding region of the gene, a 354C→T transition in exon 2, predicting a R110X premature termination, was found on his maternal allele.

### Aberrant processing of the 1270 + 1G→A mRNA

cDNA of patient 2 was amplified with PCR and the products were analysed on polyacrylamide gel electrophoresis (PAGE). Aberrant bands were eluted, reamplified and sequenced (Fig. 2). A band amplified with RAF11-RAF12 included a mutant (1270 + 1G→A) intron 7; introns 8 and 9 were spliced out correctly from this messenger. Inclusion of an intron in the mRNA is not a common effect of 5′-splice mutations on mRNA (Scott et al. 1993). However, as a consequence of the multiplicity of aberrant splicing products and the length of most introns, these messengers are easily overlooked. Two short bands amplified with RAF9-RAF12 missed exons 6 and 7; the shortest product also missed exon 9 (Fig. 2). One aberrant product amplified with primers RAF9-RAF12 was also amplified from the cDNA of most other MPS VII patients; direct sequencing showed its sequence to be identical with that of nt 253–410 of SMA 4 cDNA (Theodosiou et al. 1994). SMA 4 is a member of a family of expressed sequences consisting of various combinations of β-glucuronidase-related pseudoexons; PCR amplification of these sequences with our primers occurs accidentally when binding sites are conserved and are present in the correct orientation.

### Additional polymorphisms in exon 12

Sequencing of the PCR product amplified from genomic DNA of patient 3 showed that he was heterozygous for a A→G transition at position 2154, in the non-coding part of exon 12. Since patient 3 also carried an R374C and an S52F allele, the 2154G change could be on either allele. No material from relatives of this patient was available; therefore, we analysed position 2154 in another carrier of R374C (German patient 8 in Vervoort et al. 1996). Surprisingly, this patient was homozygous for 2154G. Thus, patient 3 and the German patient probably carry the same R374C-2154G allele and have a different MPS VII mutation on their second chromosome. We screened 30 unrelated Belgian controls for 2154G with single-track sequencing of PCR-amplified exon 12. Two individuals carried a 2154G allele (estimated allele frequency 3%). Single-track sequencing of exon 12 disclosed an additional polymorphism located 3′ of the wild-type polyadenylation site (and therefore not present in mRNA); one healthy individual was homozygous for 2243ΔGT (Fig. 1).

### Alternative polyadenylation sites

To compare the expression of the 2154G and 2154A messenger, we used 3′-RACE-PCR. 3′-RACE-PCR products were amplified from cDNA of patient 3 and of a control carrier of 2154G and directly sequenced. Sequencing results demonstrated that both alleles were almost equally represented in cDNA (Fig. 3).

When 3′-RACE products were directly sequenced, the involvement of two different polyadenylation sites was observed. The 3′ end of the first cDNA was identical with the published 3′ sequence; the second cDNA was 13 nt shorter (Fig. 3). Sequences derived from both messengers

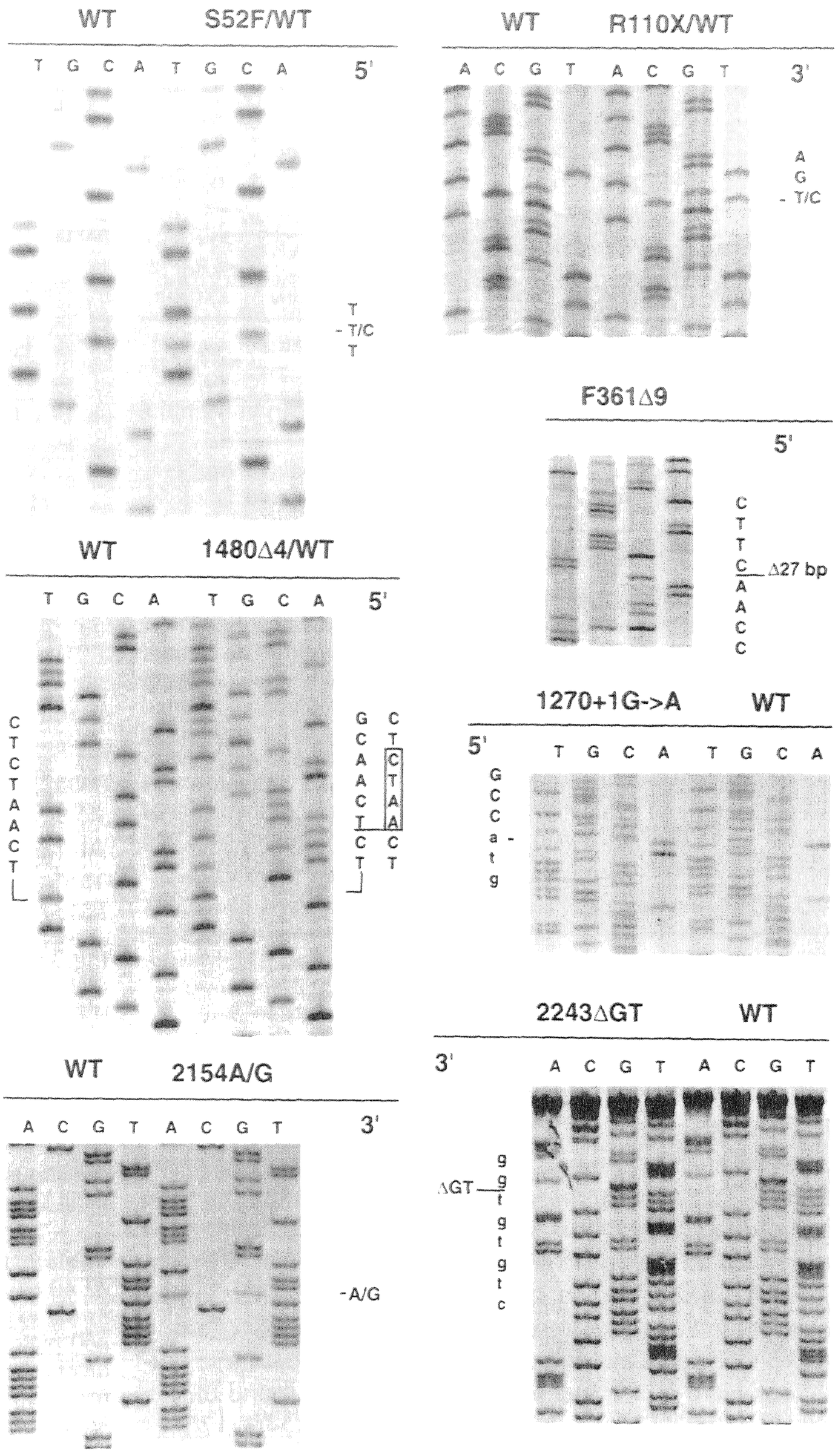


**Fig. 1** Detection of nucleotide changes in the β-glucuronidase gene. The strategy used for PCR amplification has been described previously. Results obtained after direct sequencing of PCR products amplified from patient genomic DNA (S52F, R110X, 1270 + 1G→A, 1480Δ4, 2154A/G, 2243ΔGT) or cDNA (F361Δ9) are shown. F361Δ9, 1270 + 1G→A and R110X were sequenced in one direction only; 1270 + 1G→A was confirmed unambiguously by digestion of the PCR product with restriction enzyme *Nla*III. All other changes were sequenced in both directions. *WT* Wild-type sequence

had almost equal intensity. The 2154A/G polymorphism is localized between the two potential polyadenylation signals. However, polyadenylation was not influenced by the 2154A/G allele: both sites were equally used in cDNA of individuals with 2154A/A, 2154G/G or 2154A/G alleles.

## Expression studies

To investigate the causal relationship between the S52F and the F361Δ9 change found on one patient's MPS VII allele and the observed enzyme deficiency, mutant cDNA was constructed in vitro and used to transfect COS cells transiently. After transfection, media and cells were collected, and β-glucuronidase enzyme activity was measured and compared with activity obtained after transfection with wild-type cDNA and vector only. The results clearly demonstrate that both changes completely destroy enzyme activity (Table 2). In vitro expression of the R357X and R374C mutations has been reported previously (Shipley et al. 1993; Vervoort et al. 1996). The effect of the R110X and 1480Δ4 mutations on enzyme activity was not studied, since they resulted in premature termination of translation.

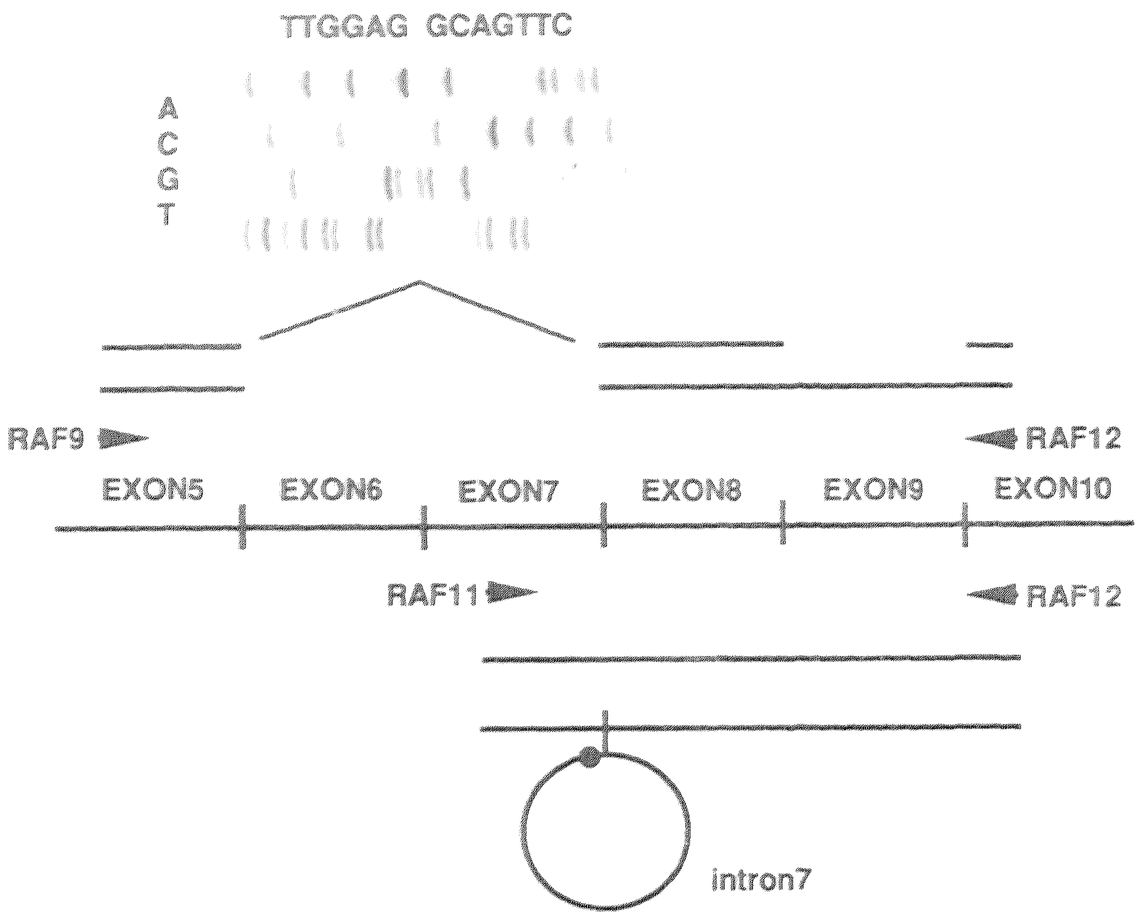


**Fig. 2** Aberrant splicing of the 1270 + 1G→A mRNA. When cDNA of a patient homozygous with respect to his genomic DNA for the 1270 + 1G→A mutation was amplified with PCR (primer pairs RAF9-RAF12 and RAF11-RAF12), bands with aberrant lengths were seen on PAGE. These bands were eluted, reamplified and sequenced; skipping of exons 6 and 7 with or without skipping of exon 9, and inclusion of the mutant intron 7 were found, whereas in normal messenger, only alternative skipping of exon 6 was seen. Skipping of exon 9 did not occur together with inclusion of intron 7. The cDNA (*centre*), the position of both primer pairs and the aberrant fragments amplified when using them are shown

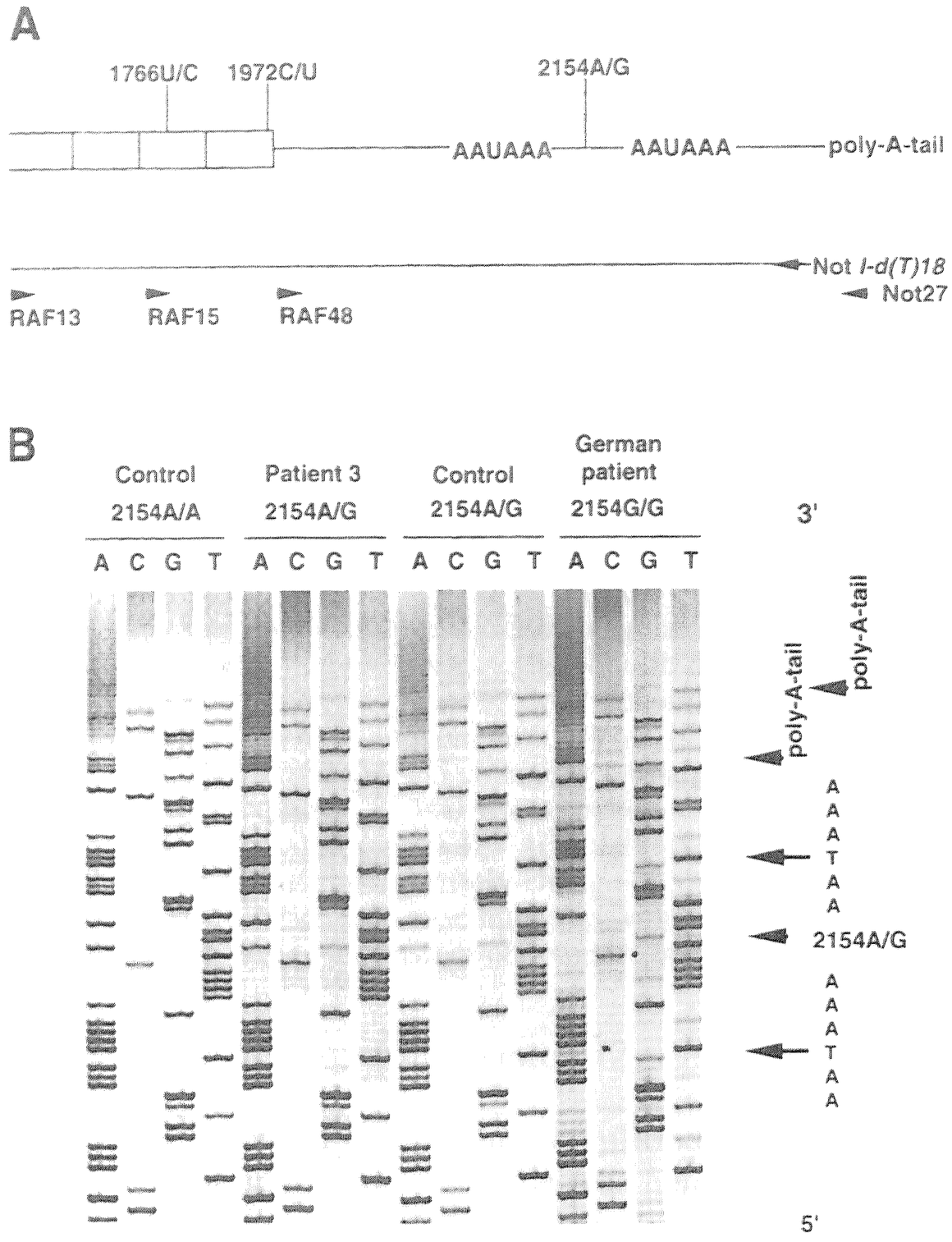


**Fig. 3A, B** 3′-RACE-PCR of cDNA. **A** Strategy used for RACE-PCR. *Top* mRNA; the coding region of the exons is *boxed*. The position of the sequence polymorphisms is indicated. *Bottom* Synthesis of the first cDNA strand is shown together with the position of the primers for PCR and sequencing (*arrows*). **B** Alternative polyadenylation of mRNA and messenger stability are not influenced by the 2154A/G allele. Sequencing results obtained with primer RAF48 are shown. The positions of the polyadenylation consensus sequences, the polyadenylation sites (*arrows*) and 2154A/G polymorphism are indicated

## Discussion

### Mutation analysis

Analysis of the β-glucuronidase gene of MPS VII patients has revealed extensive genetic heterogeneity, even within a homogeneous group of patients presenting with hydrops fetalis. However, an increasing number of mutations have been found in more than one family, especially in families of related ethnic origin (A619V in Japan, L176F in Spain, R477W in Arabs). In the present study, another three changes have been demonstrated in more than one family (R374C, R357X and F361Δ9). It is unlikely that these mutations have arisen independently. They are found on the same haplotype background (1766C-1972T-2154G, 1766C-1972C and 1766C-1972C, respectively) in families originating from neighbouring countries (Germany-Czechoslovakia, The Netherlands-Belgium and The Netherlands-Belgium, respectively). As a consequence, screening for previously described (unique) MPS VII mutations might simplify molecular analysis in patients of related ethnic origin.

To date, four small deletions have been found in the β-glucuronidase gene: the 1107Δ27, 1480Δ4 and 1900ΔGA deletions on MPS VII alleles, and the 2243ΔGT polymorphism. Cooper and Krawczak (1993) have studied large series of small deletions in human genes and discuss possible mutational mechanisms. Small direct repeats flanking or overlapping all small deletions are consistent with a slipped mismatch repair mechanism. Increased frequencies of some tri- and tetranucleotides surrounding small deletions have also been found. The deletions in the β-glucuronidase gene are in good agreement with the conclusions of Cooper and Krawczak (Fig. 4).

**Table 2** Expression of normal and mutant enzymes in COS cells. At 60 h after transfection with wild-type or mutant cDNA cloned in the expression vector pJC119, COS cells were collected from p60 plates and lysed in 0.5% deoxycholic acid. Cell lysates and media were assayed for β-glucuronidase activity by using 4-methylumbelliferyl-β-D-glucuronide (1 unit is the amount that releases 1 nmol 4-methylumbelliferone/h). The endogenous activity was measured in parallel COS cells transfected with expression vector without the insert. Each construct was transfected in duplicate

| Construct | β-Glucuronidase activity (units/mg cell protein minus endogenous) | | |
|---|---|---|---|
| | Cell | Media | Total |
| Wild type | 1542 | 1400 | 2942 |
| S52F | 21 | 0 | 21 |
| F361Δ9 | 0 | 0 | 0 |

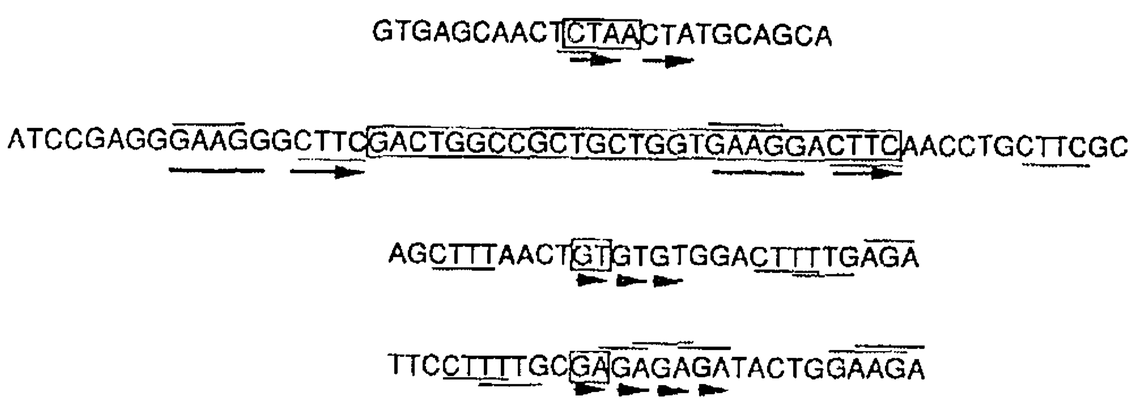


**Fig. 4** Sequence environment of small deletions. The sequence surrounding the four small deletions is shown (nucleotides 1480Δ4, 1107Δ27, 2243ΔGT and 1900ΔGA). The deleted sequence is *boxed*; direct repeats are *underlined* with *bold arrows*. Sequences found with increased frequency near small deletions (Cooper and Krawczak 1993) are *underlined*; sequences in the antisense strand are *overlined*

### Nonsense mutations affecting mRNA quantity

Although the RT-PCR method used here provides no information on the absolute levels of mRNA expressed, it allows a comparison of mRNA levels synthesized from both alleles of a heterozygous patient. Analysis of the cDNA of patients 1, 4 and 5 has revealed only one mutant allele. Analysis of genomic DNA is required to detect the mutation on the second allele, which has subsequently been confirmed to be absent in the cDNA (R357X, R110X, 1480Δ4); thus, these alleles produce greatly reduced levels of stable mRNA (RNA-). The present observations fit the translation-coupling model of Urlaub et al. (1989), a model that explains the 5′-to-3′ gradient of the influence of nonsense mutations on mRNA levels. Indeed, the R110X allele, mutated in exon 2, and the 1480Δ4 frameshift, resulting in a premature stop codon in exon 10, are RNA- alleles. It is interesting to note that 1107Δ27, which does not affect the reading frame, produces higher (and probably normal) levels of mRNA than that found in R357X and 1480Δ4.

### Alternative polyadenylation in fibroblasts

Sequencing of 3′-RACE-PCR products has disclosed the use of two alternative polyadenylation sites in fibroblasts. The longest messenger corresponds to the published cDNA (Oshima et al. 1987), the second is 10–13 nt shorter (Fig. 3). Both 3′ termini are amplified in equal amounts. Equal usage of both polyadenylation sites is not restricted to fibroblasts: it has also been observed when cDNA of lymphoblastoid cell lines is amplified (results not shown). Two consensus AATAAA signals are present near the 3′ end of the β-glucuronidase cDNA. Both cleavage sites are separated by 7–10 bp and 24 bp from the second signal. Since the usual distance between the signal and the polyadenylation site is 10–30 bp, only the second AATAAA signal may be functional.

The 2154A/G change is localized between the two polyadenylation signals. Nucleotide changes affecting polyadenylation are generally found in the consensus sequence; however, mutation of surrounding nucleotides might affect the efficiency of polyadenylation and, as a consequence, affect mRNA levels in some cases (Cai et al. 1992). Studies of mammalian polyadenylation have demonstrated the importance of downstream or upstream efficiency elements (for a review on polyadenylation, see Manley 1995). Therefore, we have compared the expression of both 2154 alleles in two heterozygotes. Since equal amounts of both alleles are expressed, we conclude the 2154A/G change does not affect mRNA stability. Furthermore, we have observed no influence of the 2154 haplotype on alternative polyadenylation in homozygotes. These results, together with the relatively high allele frequency (3% in controls), demonstrate that the 2154A/G change is probably a harmless polymorphism.

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