Detec\tion of Point Mutations and a Gross Deletion in Six Hunter Syndrome Patients

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We have used screening with the polymerase chain reaction and chemical mismatch detection of amplified cDNA to detect and characterize deletions and point mutations in six Hunter Syndrome patients. A high degree of mutational heterogeneity was observed. The first patient is completely deleted for the gene coding for \( \alpha-L \)-iduronate sulfate sulfatase, while the second has a point mutation that creates a stop codon. The third patient shows a point mutation that creates a novel splice site that is preferentially utilized and results in partial loss of one exon in the RNA. Patients 4, 5, and 6 have point mutations resulting in single amino acid substitutions. Four of the six single-base changes observed in this study were examples of transitions of \( \text{CpG} \) to \( \text{TpG} \). This study has demonstrated a procedure capable of detecting all types of mutation that affect the function of the IDS protein and should enable direct carrier and prenatal diagnosis for Hunter syndrome families. 

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INTRODUCTION

Hunter syndrome or mucopolysaccharidosis type II (MPS II) is an X-linked recessive disorder caused by an absence or reduction of the lysosomal housekeeping enzyme \( \alpha-L \)-iduronate sulfate sulfatase (IDS). This leads to accumulation of the incompletely degraded mucopolysaccharides heparan and dermatan sulfate (McKusick and Neufeld, 1983; Spranger, 1984; Benson and Fensom, 1985; Bielicki \textit{et al.}, 1990). Symptoms of the disease include short stature, coarse facial features, stiff joints, progressive deafness, and mental retardation. Heart disease, resulting from a combination of valvular, myocardial, and ischemic factors, is the usual cause of death, and severely affected males die before adulthood, although milder cases have reached their eighth decade and have been capable of reproduction (Hobolth and Pedersen, 1978). It has been estimated that the incidence of Hunter syndrome is 7.5 per million male live births (Young \textit{et al.}, 1982), but a higher number (14.8 per million) of male live births has been proposed for the Ashkenazi Jewish population (Schaap and Bach, 1980).

Clinical diagnosis is based upon measuring IDS activity in peripheral white blood cells and serum and/or detecting accumulation of glycosaminoglycans in urine (Benson and Fensom, 1985). Carrier status is presently determined by enzyme assay for IDS in blood samples or preferably hair roots (Yutaka \textit{et al.}, 1978; Archer \textit{et al.}, 1982; Chase \textit{et al.}, 1986). Enzyme assays in blood samples, however, have been shown to be ambiguous in at least 15\% of obligate carriers (Zlotogora and Bach, 1984). Better results can be obtained by hair root analysis (Chase \textit{et al.}, 1986), but absolute certainty is not achieved. Also, linked restriction fragment length polymorphisms (RFLPs) do not provide definite carrier diagnosis, and therefore more accurate and direct methods for carrier detection are required. These must be based on the direct detection of the gene defect as illustrated for example by work on hemophilia B (Green \textit{et al.}, 1989; 1991a,b; Montandon \textit{et al.}, 1989).

A full-length cDNA clone of 2.3-kb coding for the human IDS gene has recently been cloned and sequenced (Wilson \textit{et al.}, 1990). An open reading frame of 1650 bp was identified. The predicted 550 residue amino acid sequence includes 25 residues of signal sequence and 8 residues of propeptide sequence. As pointed out by Wilson \textit{et al.} (1990), human IDS shares homology with several other sulfatase enzymes, including human arylsulfatases A, B, and C, human glucosamine-6-sulfatase, and sea urchin arylsulfatase (Ye et al., 1987; Robertson \textit{et al.}, 1988; Sasaki \textit{et al.}, 1988; Stein \textit{et al.}, 1989a,b; Peters \textit{et al.}, 1990). The IDS cDNA clone has been used as a probe to screen 23 severely affected Hunter syndrome patients for gene deletions and rearrangements (Wilson \textit{et al.}, 1990). Seven patients were found to have a deletion or rearrangement, and further analysis of these showed that two patients had deletions that removed the entire IDS gene. The remaining patients had either small deletions or point mutations, which were not detected by the method used.

Here we describe the characterization of the Hunter syndrome mutation in each of a group of six patients analyzed using an approach that detects all types of mutations that affect the structure and/or stability of the
### MATERIALS AND METHODS

#### DNA and RNA extractions

Genomic DNA and RNA were prepared together from either peripheral lymphocytes or cultured fibroblasts using the acid-guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987).

#### Reverse transcription and PCR amplification

RNA (200 to 500 ng) was reverse transcribed prior to PCR amplification. A total of 50 ng of the appropriate primer was annealed to 200-500 ng of RNA in a volume of 10 μl TE buffer at 65°C for 10 min. After snap cooling on ice, reactions were made up to 20 μl with 4 μl 5× reverse transcriptase buffer (BRL), 5 μl 5 mM dNTPs, 25 units RNase inhibitor (BRL), and 20 μl of cDNA was amplified using the conditions described above. Outer primers (see Fig. 2 and Table 1) were used for 30 cycles of PCR amplification of genomic DNA. Genomic DNA was amplified in the first instance using primer pairs specific to the 5' and 3' ends of the gene coding for IDS (IDS C/41 and IDS D/E, respectively) (see Table 1). All PCRs were in a total volume of 50 μl containing 67 mM Tris–HCl, pH 8.8, 16.6 mM ammonium sulfate, 6.7 mM magnesium chloride, 170 μg/ml bovine serum albumin, and 10 mM 2-mercaptoethanol, 30 ng of each primer, 2.5 units AmpliTaq Taq polymerase (Perkin–Elmer/Cetus), and approximately 10–20 ng of DNA template. Thirty cycles of amplification were carried out (93°C/1 min, 60°C/1 min, 72°C/3 min). The polyadenylation region was also amplified from genomic DNA using the primers pAd 1 and pAd 2 (see Table 1). Conditions used were as described above and amplification was for 30 cycles of 93°C/1 min, 58°C/1 min, 72°C/3 min. Products of all PCR reactions were separated on 2.5% agarose gels and purified from gels using genclean (Bio 101). Then, 100–200 ng of gel-purified reactions were sequenced by the dideoxy chain termination method (Sanger et al., 1977; Green et al., 1989).

#### Primer Sequences and Sizes of Expected Products for Primer Pairs Used in Amplification of the IDS Sequence

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Primer 1 (5’~3’)</th>
<th>Position (5’~3’)</th>
<th>Primer 2</th>
<th>Position (5’~3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDS C/L</td>
<td>CTGCTAACTGCGCACCCTGC</td>
<td>60–79</td>
<td>TTGAGCTTCTCCGTTG</td>
<td>966–938</td>
<td>896</td>
</tr>
<tr>
<td>IDS V/N</td>
<td>GAAGCGGCGGCGCTCGAG</td>
<td>102–119</td>
<td>GATGCTTGTTGTCCTACATCGAG</td>
<td>941–921</td>
<td>840</td>
</tr>
<tr>
<td>IDS O/B</td>
<td>GCTGACAAACAGACAGCCTG</td>
<td>712–731</td>
<td>CGGACACCTCACTCCTCC</td>
<td>1850–1831</td>
<td>1139</td>
</tr>
<tr>
<td>IDS P/D</td>
<td>CTGAGCAACGCCATACAGTTG</td>
<td>720–746</td>
<td>GCTGAAAGGAGGACACATCAC</td>
<td>1822–1803</td>
<td>1092</td>
</tr>
<tr>
<td>IDS E/D</td>
<td>GCTTGGCTTCCATCTGATG</td>
<td>1630–1649</td>
<td>GCTGGAAGGAGGACACATCAC</td>
<td>1822–1803</td>
<td>193</td>
</tr>
<tr>
<td>IDS C/41</td>
<td>GCCTGACTCGCCACCCCTGC</td>
<td>60–79</td>
<td>GACGGGCTCGACACACCAGACC</td>
<td>184–164</td>
<td>105</td>
</tr>
<tr>
<td>IDS pAd1/2</td>
<td>GTGATGTGCTCCTCCAGCG</td>
<td>1803–1822</td>
<td>GGTCTTCTGATCAAAGAGC</td>
<td>2095–2078</td>
<td>283</td>
</tr>
</tbody>
</table>

*Primers were designed from sequence data provided by Dr. Hopwood.*

### Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Band size (bp) shown by AMD</th>
<th>Mutation in cDNA</th>
<th>Mutation confirmed in genomic DNA</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Entire IDS gene and proximal marker VK21c deleted. Amino acid change.</td>
</tr>
<tr>
<td>2</td>
<td>A 550 nt (H + O)</td>
<td>C&lt;sub&gt;550&lt;/sub&gt; to T</td>
<td>Yes</td>
<td>Arginine&lt;sub&gt;550&lt;/sub&gt; becomes a translation stop codon.</td>
</tr>
<tr>
<td>3</td>
<td>B 500 nt (H + O)</td>
<td>60bp of cDNA lost (G&lt;sub&gt;1944&lt;/sub&gt;-G&lt;sub&gt;1950&lt;/sub&gt;)</td>
<td>C&lt;sub&gt;1946&lt;/sub&gt; to T transition</td>
<td>Silent change in codon creating a cryptic donor splice site leading to partial loss of an exon. Serine&lt;sub&gt;389&lt;/sub&gt; to leucine substitution changes a conserved residue within a region of homology among other sulfatases (see Fig. 5). Tryptophan&lt;sub&gt;507&lt;/sub&gt; to serine substitution results in the introduction of a small polar side chain in a hydrophobic domain.</td>
</tr>
<tr>
<td>4</td>
<td>B 400 nt (H + O)</td>
<td>C&lt;sub&gt;1122&lt;/sub&gt; to T</td>
<td>Yes</td>
<td>Threonine&lt;sub&gt;249&lt;/sub&gt; silent change: polymorphism or private mutation. Proline&lt;sub&gt;350&lt;/sub&gt; to arginine substitution.</td>
</tr>
<tr>
<td>5</td>
<td>B 200 nt (H)</td>
<td>G&lt;sub&gt;1826&lt;/sub&gt; to C</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A 460 nt (H)</td>
<td>C&lt;sub&gt;561&lt;/sub&gt; to T</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>A 500 bp (H + O)</td>
<td>C&lt;sub&gt;603&lt;/sub&gt; to G</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

*Note. A, B, sections of amplified cDNA representing 5' and 3' portions, respectively, of mRNA. H, hydroxylamine; O, osmium tetroxide.*
amplification (93°C/1 min, 58°C/1 min, 72°C/5 min). First-round PCR products (5 µl) were amplified for a further 30 cycles as before using the inner primers (Fig. 2, Table 1). Products were separated on 1% agarose gels and extracted using Geneclean (Bio 101).

Chemical mismatch detection analysis and sequencing. Two hundred nanograms of each purified PCR product (with the exception of those representing the polyadenylation region, which were sequenced directly) were screened for mutations using chemical mismatch detection (Montandon et al., 1989; Cotton et al., 1988). DNA was annealed in a 10:1 ratio with a homologous end-labeled probe. Any differences between the strands of these heterodimers lead to mismatched cytosine (C) or thymine (T) residues, which are modified by hydroxylamine (2.3 mM) or osmium tetroxide (0.025%), respectively, for 2 h at 37°C. Cleavage at the modified sites is then carried out with piperidine (1 M) for 30 min at 90°C. Products were resolved on 6% acrylamide denaturing gels and autoradiographed. Regions shown to contain mismatches were sequenced by the dideoxy chain termination method (Sanger et al., 1977; Green et al., 1989).

RESULTS

The mutations in the DNA and RNA of six unrelated patients with severe Hunter syndrome were obtained (see Table 2). The mutations in each case were characterized as follows. The genomic DNA of every patient was initially screened by PCR reactions specific to the 5' and 3' ends of the gene. These were directed by primer pairs IDS C/41 and IDS D/E, respectively (Table 1). Patient 1 was negative for both reactions (see track 1,
Figs. 1a and 1b) and was also negative when tested by a PCR reaction detecting the anonymous proximal marker VK21C (DXS269) (Suthers et al., 1989) (results not shown). However, the sample was positive for an STS representing U6.2 (DXS304) (Rousseau et al., 1990), which showed the ability of the DNA to act as a template for amplification. Patient 1 therefore presumably has a deletion of the whole IDS gene extending proximally at least as far as DXS269 in Xq27.3.

The remaining five patients were positive for the 5'- and 3'-specific PCR reactions (see Figs. 1a and 1b). To screening these patients for point mutations, total RNA was first extracted from primary fibroblast cultures. The IDS mRNA was reverse transcribed in two overlapping sections using primers IDS L and IDS B for the 5' (A) and 3' (B) sections, respectively, and then amplified by two nested PCR reactions using the primer pairs IDS C/L and IDS V/N for section A and IDS O/B and IDS P/D for section B (Table 1 and Fig. 2). The polyadenylation region was amplified from genomic DNA and sequenced directly without detection of any significant abnormality. The overlapping, amplified cDNA sections A and B were then subjected to chemical mismatch analysis to identify and locate any point mutation or mRNA abnormality. The mutations are summarized in Table 2.

Mismatch analysis of patients 2-4 and 6 resulted in detection of a single mismatch band in either reaction A or B in each case. Such a band could be elicited however by either hydroxylamine or osmium tetroxide (see for example patient 3 reaction B, track 5, Fig. 3). In patient 5, two mismatch bands were detected, one in each section of the cDNA (see Fig. 3, track 3 in the hydroxylamine reaction for the band in reaction B; result not shown for reaction A).

To characterize fully the cDNA defects revealed by the mismatch analysis the appropriate segments of the amplified section A and/or B were sequenced directly using either PCR or ad hoc primers. This revealed that in patient 2, the mismatch band of 550 nt in section A was due to a C → T transition at residue 698, which converted the Arg698 codon into a translation stop signal (Fig. 4a, Table 2). The mutation was confirmed by sequence analysis of the coding region amplified from genomic DNA using a primer derived from an intron sequence paired with a cDNA-specific primer.

In patient 3 the 500-nt band seen with both chemicals in section B is due to the loss of 60 bp of cDNA extending from nucleotide position 1244 to 1305 (Table 2, Figs. 4b and 5). Analysis of this region of the patients' genomic DNA showed that the sequence absent from the cDNA was present in genomic DNA, but that a C → T transition had occurred in a nucleotide corresponding to position 1246 of the cDNA (the sequence change in the antisense strand is shown in Fig. 4c). This does not alter the amino acid sequence but creates a donor splice site (CAGGGAGAGA → CAGGTGAGA). As a result, part of this exon [nts 1245–1304 of the cDNA (Wilson et al., 1990)] is spliced out in the mature mRNA along with the normal intron sequence prior to nucleotide 1305. To demonstrate the preferential use of the novel splice site in patient 3, the size of the PCR product from reaction B was compared with that of a normal sample. The amplified cDNA derived from the patient consisted exclusively of a shorter product (1032 nts; see track 2, Fig. 6) compared to the normal control. Existence of an exon boundary following nucleotide 1304 of the cDNA was confirmed by sequencing normal genomic DNA (unpublished results).

In patient 4, the mismatch band of 400 nt in section B is caused by a C → T transition at nucleotide 1122 that was also found in genomic DNA using a primer complementary to nearby intron sequence and results in substitution of serine66 by leucine (Fig. 4d, Table 2).

In patient 5 the mismatch band of 460 nt in section A is due to a C → T transition at nucleotide position 562 (seen in both genomic and cDNA), which causes a silent change in the codon for threonine53 (ACC to ACT). The second mismatch band in this patient, the 200-nt band in section B, is due to a G → C transversion at nucleotide 1629. This causes the substitution of tryptophan53 by serine (Fig. 4e, Table 2), a change that was confirmed in genomic DNA.

Finally, in patient 6 the mismatch band of 500 nt in section A is due to a C → G transversion at position 603 that converts Pro160 to Arg (Fig. 4f, Table 2), as also demonstrated in genomic DNA.
FIG. 4. Sequence data relating to observed mutations. (a) Track 1, patient sample; track 2, normal control. (b–e) Track 1, normal control; track 2, patient sample. (a) C → T transition at nt 638 of patient 2; (b) novel junction seen in the coding sequence of patient 3, where G1246 and G205 are spliced together (arrowed); (c) antisense strand of genomic DNA in patient 3 showing G → A transition at nt 1246 (arrowed) and new exon 3' end (underlined); (d) C → T transition at nt 1122 (arrowed) of patient 4; (e) G → C transversion at nt 1629 (arrowed) of patient 5; (f) C → G substitution at nt 603 (arrowed) of patient 6.

DISCUSSION

In every one of the six patients analyzed, a mutation was found that can be considered the cause of the disease. This is beyond any doubt in patient 1, where the whole gene was deleted and in patient 2, where a stop codon at position 172 causes the functional loss of most of the coding sequence. In patient 3, 20 amino acids are lost from a relatively well-conserved region of the sulfatases, and this also represents a gross abnormality.
FIG. 5. Events leading to partial loss of an exon in the cDNA of patient 3. Exons are boxed and exon and intron sequences are shown in upper- and lowercase letters, respectively. Nucleotides 1304 and 1305 represent the exon junction in wildtype cDNA. The site of mutation at nucleotide 1246 is marked by an asterisk. The $C_{1246} \rightarrow T$ transition in the patient DNA creates a donor splice site that converts nucleotides 1245–1304 of the cDNA into an intron sequence.

within the coding sequence of the gene. Less certain are the functional consequences of the single amino acid substitutions observed in patients 4, 5, and 6. However, the Ser$_{325} \rightarrow$ Leu substitution of patient 4 represents a nonconservative change (small polar to large hydrophobic) of a residue in a region that is conserved in many sulfatases (see Fig. 7). Similarly the Trp$_{502} \rightarrow$ Ser substitution of patient 5 represents a major change in the properties of the amino acid side chain. Thus the very large

FIG. 6. Preferential use of the novel splice site in patient 3. M, 1-kb ladder size marker; tracks 1 and 2, reaction B product from a normal control and patient 3, respectively.

FIG. 7. Diagram of the amino acids 327 to 336 of human IDS (top line) and below, its aligned homologues: human glucosamine-6-sulfatase, human arylsulfatases A, B, and C, and sea urchin arylsulfatase, respectively (Robertson et al., 1988; Stein et al., 1989a/b; Peters et al., 1990; Yen et al., 1987; Sasaki et al., 1988). The arrow indicates the position of serine$_{325}$ substituted by leucine in patient 4. Absolutely conserved residues are boxed.
hydroporphic group of the tryptophan is replaced by the small polar serine side chain. This change occurs in a very hydrophobic domain (WVGVF → VSFGVGF) and is therefore likely to cause a significant reduction in the stability or activity of the enzyme. This latter patient also shows a translationally silent change that is expected to be neutral. Whether this represents a common polymorphism is not yet known. Finally, the Pro160 → Arg substitution of patient 6 is also a nonconservative change, which in this case involves the amino acid proline, which frequently has important structural roles.

It is interesting to note that four out of six of the single nucleotide changes described in this study were C → T transitions occurring at the CpG dinucleotide (C_{635} → T in patient 2, C_{1246} → T in patient 3, C_{112} → T in patient 4, and C_{560} → T in patient 5). This result agrees with earlier observations that the CpG dinucleotide is a “hotspot” for mutations within the mammalian genome (Bird, 1980; Green et al., 1990).

The procedure that we used to detect the mutation of the above patients makes use of amplified cDNA. This reduces the work required to characterize the mutation because the mRNA presents the coding sequence in a continuous stretch, a property that has also been invaluable in the characterization of the mutations of large and complex genes, such as those for factor VIII and dystrophin (Naylor et al., 1991; Roberts et al., 1991). Thus two nested PCR reactions applied after first-strand cDNA synthesis allow the isolation of the whole coding sequence of the IDS gene as two purified and abundant overlapping segments. A total of four chemical mismatch reactions then identify and locate any mutation, thus reducing to a minimum the sequencing required to fully characterize the mutation. Another advantage of analyzing sequence changes at the level of the cDNA is that the effects of mutations on RNA splicing may be directly identified. This is clearly illustrated by patient 3, where a single base substitution in an exon creates a new donor splice site and causes the loss of 60 nt from the message. Analysis of the patient cDNA (see Fig. 6 and Results) clearly indicates that the new donor site is the functional site in patient 3, a fact that could not have been definitely predicted from the knowledge of the single base substitution observed in genomic DNA because the rules governing mRNA splicing are not yet well defined. Furthermore, the findings in patient 3 are in keeping with the “exon definition” hypothesis (Robberson et al., 1990). According to this hypothesis the spliceosome begins to assemble at the 3' end of an intron, followed by a search for a downstream donor splice that together with the 5' acceptor site defines the exon. The new donor splice site in patient 3 may therefore be preferred to the natural splice site because it is closer than the latter to the preceding acceptor site.

Analysis of genomic DNA is required to confirm the mutation seen in patients cDNA, especially as illustrated for patient 3. Additionally, a PCR reaction was used to analyze the cleavage polyadenylation signal region of the gene, since mutations of these sites are known to be detrimental in the globin genes (Orkin et al., 1985). The promoter should also be analyzed in genomic DNA, but such a region of the IDS gene has not been defined yet and we have not examined it.

In conclusion, we have characterized the mutation of 6 patients with Hunter syndrome by a procedure that should be capable of detecting most types of mutations that affect function and/or stability of the IDS protein or mRNA. The efficiency of mutation detection relies on the ability of the chemical mismatch procedure to detect all types of point mutation. From previous studies in hemophilia B (Green et al., 1991a, and unpublished), the mutation in 160 out of a total of 161 unrelated patients with the disease was identified using this approach. The one patient in whom no mutation was detected by mismatch analysis was shown by complete sequence analysis to lack a mutation within the essential regions of the factor IX gene. This indicated that the inability to find the mutation was not due to failure of the chemical mismatch technique.

The method described above should allow carrier and prenatal diagnosis based on the direct detection of the gene defect according to the strategy previously developed for hemophilia B (Green et al., 1991b).

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We are grateful to Professor M. Bobrow for constant support; to Dr. J. J. Hopwood for kindly sending us the cDNA sequence of the IDS gene prior to publication; to Professor M. Adinolfi for help in obtaining patient details and samples; and to Miss T. Slade for help in preparing patient fibroblast samples. We are also grateful to Miss A. Coffey for helpful discussions and to Ms. A. Knight for secretarial assistance. This work was supported by Wellcome Trust Grant 030850/1.5, the Waldburg Trust, the Spastics Society, and the Generation Trust.

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