

A novel missense mutation in lysosomal sulfamidase is the basis of MPS III A in a spontaneous mouse mutant

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Sanfilippo syndrome type III A (Mucopolysaccharidosis (MPS) III A) is a rare, autosomal recessive, lysosomal storage disease, characterized by the accumulation of heparan sulfate and the loss of function of lysosomal heparan *N*-sulfatase activity. The disease leads to devastating mental and physical consequences and a mouse model that can be used to explore gene therapy and enzyme or cell replacement therapies is needed. We have previously identified a mouse with low sulfamidase activity and symptoms and pathologies typical of MPS III A (Bhaumik, M., Muller, V. J., Rozaklis, T., Johnson, L., Dobrenis, K., Bhattacharyya, R., Wurzelmann, S., Finamore, P., Hopwood, J. J., Walkley, S. U., and Stanley, P. [1999] A mouse model for mucopolysaccharidosis type III A (Sanfilippo syndrome). *Glycobiology* 9, 1389–1396). We now show that the sulfamidase gene of the MPS III A mouse carries a novel mutation (G91A) that gives an amino acid change (D31N) likely to interfere with the coordination of a divalent metal ion in the active site of this sulfatase. This spontaneous mouse mutant is an excellent model for MPS III A in humans as this disease often arises due to a missense mutation in lysosomal sulfamidase.

Key words: Sanfilippo syndrome/MPS III A/sulfamidase/point mutation

Introduction

Sanfilippo syndrome belongs to the group of mucopolysaccharide (MPS) storage diseases in which lysosomal enzymes that degrade glycosaminoglycans are deficient or missing. In the normal course of events, lysosomal proteases degrade proteoglycans to give the glycosaminoglycans dermatan sulfate, heparan sulfate, keratan sulfate, and chondroitin sulfate which are themselves degraded by specific lysosomal hydrolases (Neufeld and Muenzer, 1995). Sanfilippo syndrome, or muco-

polysaccharidosis type III, arises from an inability to degrade heparan sulfate leading to storage of heparan sulfate in lysosomes (Neufeld and Muenzer, 1995). There are four subtypes of MPS III (A, B, C, and D) in which different heparan sulfate degrading hydrolases are deficient. MPS III A arises from the loss of activity of the *N*-sulfatase termed lysosomal sulfamidase (EC 3.10.1.1) that removes sulfate from glucosamine sulfate in heparan sulfate (Kresse, 1973). The prevalence of a particular subtype varies with geographical region, MPS III A being the most common subtype in the United Kingdom (Whiteman and Young, 1977) and in the Netherlands (Poorthuis *et al.*, 1999). To date approximately 42 mutations have been described in the sulfamidase gene of MPS III A patients from different geographical regions (Scott *et al.*, 1995; Bhaumik *et al.*, 1999; Bunge *et al.*, 1997; Weber *et al.*, 1997; Di Natale *et al.*, 1998; Montfort *et al.*, 1998; Esposito *et al.*, 2000), and about 50% are missense point mutations.

MPS III A has been reported in the dog (Fischer *et al.*, 1998), and we previously identified a mouse with MPS III A that replicates many of the histopathological features found in MPS III A in children (Bhaumik *et al.*, 1999). Mice of this strain die at about 10 months of age exhibiting a distended bladder and hepatosplenomegaly. In brain sections from these mice, distended lysosomes, some with typical zebra body morphology, and many containing periodic-acid Schiff positive storage material, are prevalent. Urinalysis revealed an accumulation of heparan sulfate that contained glucosamine-*N*-sulfate at the non-reducing end and was susceptible to digestion with recombinant sulfamidase. Assays of a variety of lysosomal hydrolases in brain, liver, and kidney extracts uncovered a specific defect in sulfamidase activity. Interestingly, the activity was reduced by about 97% but was not completely absent, suggesting a point mutation as the basis of the mouse MPS III A phenotype. In this paper, we identify a missense mutation in the coding region of the sulfamidase gene, and show that this provides the molecular basis of MPS III A in our spontaneous mouse mutant.

Results

A missense mutation in the sulfamidase gene of mice with MPS III A

In order to identify the molecular basis of the MPS III A mouse, primers were designed based on mouse (Costanzi *et al.*, 2000) and human (Scott *et al.*, 1995) sulfamidase cDNA sequences, and poly(A)⁺ RNA from livers of affected and unaffected mice was reverse transcribed and amplified to obtain the ~1.6 kb sulfamidase gene coding region. Sequence

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analyses of reverse transcriptase (RT)-PCR products revealed a point mutation (G to A) at nucleotide position 91 with a corresponding amino acid change from aspartic acid to asparagine (D to N) at position 31, as the only difference between mutant and wild type cDNAs (Figure 1). The G91A point mutation was also confirmed by sequencing PCR products from genomic DNA of wild type and mutant mice. The C57Bl/6 mouse sulfamidase coding sequence is identical to the wild type sequence from our strain of mice (background mainly CD1/129^{Svj}), except at amino acid position 478 where three nucleotide changes (GGG to CCC) encode for a P instead of the G found in the C57Bl/6 sequence (Figure 2). Interestingly, P478 is conserved in the human sulfamidase gene sequence (see Figure 3). It is not known whether the change to a G at this position in C57Bl/6 mice has an effect on sulfamidase activity.

The MPS III A mutation is recessive

A method to genotype MPS III A mice based on the single point mutation was developed (Figure 1). A sulfamidase gene

fragment of 105 bp, including a portion of intron sequence and spanning the point mutation in exon 2, was amplified, and purified PCR products were digested with MspAII and separated on a 3.5% agarose gel. The point mutation abolishes the MspAII site. Therefore, the 105 bp PCR product from mutant mice remained undigested while 90 and 15 bp products were obtained from wild type mice (Figure 1). The 15 bp fragment is not shown. Sequencing of PCR products confirmed their identity. Heterozygous mice gave the two predicted bands, and the recessive nature of the mutation was confirmed by genotyping a litter from a cross between heterozygous parents (Figure 1).

Origin of the MPS III A mouse

The MPS III A mouse was initially identified in a colony of mice generated from an embryonic stem cell clone, WW6.186, that carries a targeted mutation in one allele of the *Mgat3* gene (Bhaumik *et al.*, 1999). Thus, it was possible that either the original embryonic stem (ES) cell line, or the targeted clone derived from it, had acquired a mutation in the sulfamidase

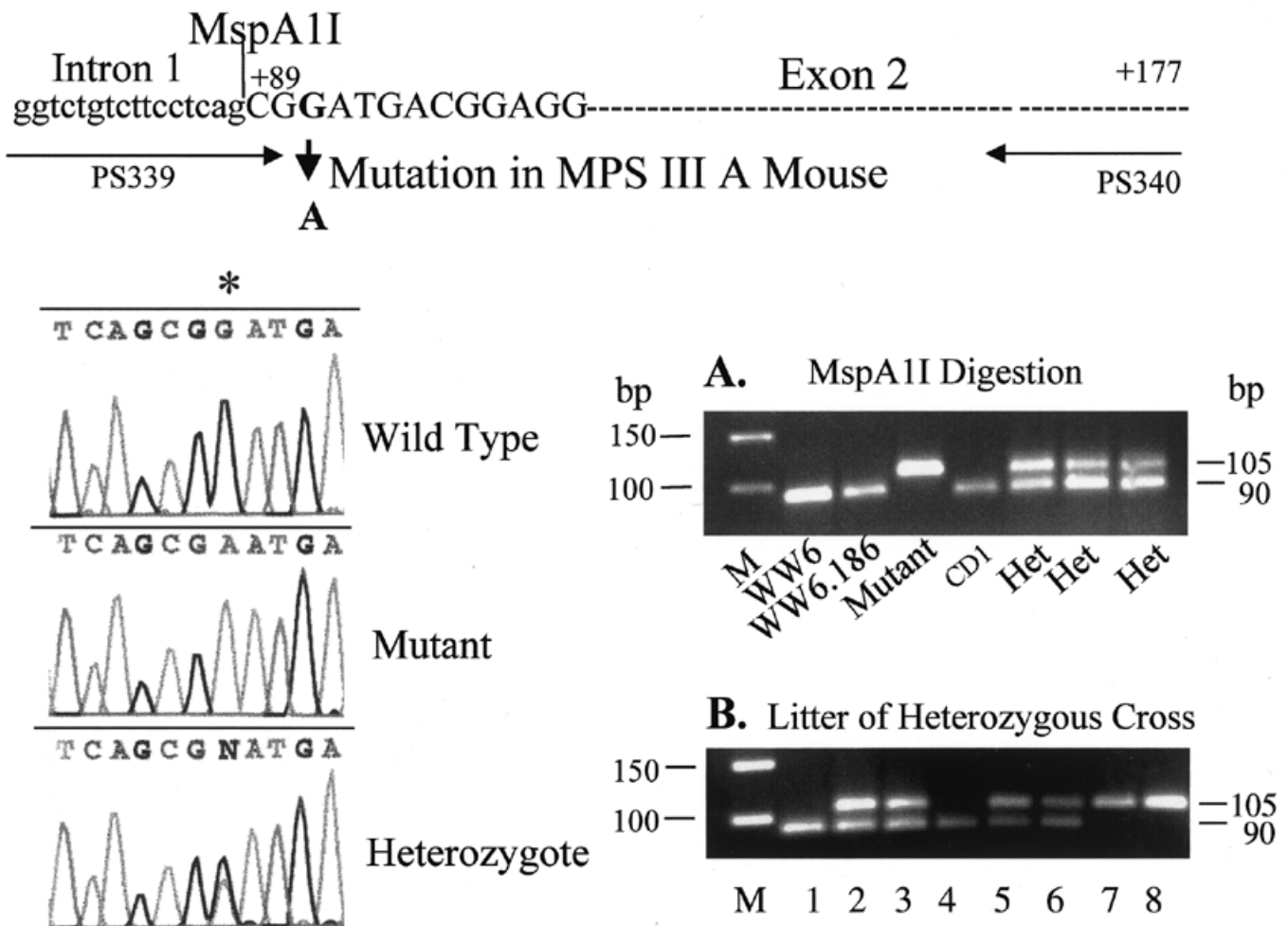


Fig. 1. The MPS III A mouse has a point mutation in the sulfamidase gene coding sequence. The diagram shows the site of the sulfamidase point mutation (G91A) and the primers used to genotype. Sequence profiles in the region of the mutation are also shown. (A) Digestion of the 105 bp PCR product by MspAII gives 90 bp and 15bp (not shown) products from wild type mice and no digestion of cDNA from mutant mice. Ten CD1 mice from Charles River were wild type (one result shown), and embryonic stem cells WW6 and WW6.186 were also wild type at the sulfamidase locus. (B) Genotype of a complete litter from a heterozygous mating, wild type, 1 and 4; mutant 7 and 8; heterozygous, 2, 3, 5, 6.

WildType	MHCPGLACCTILLVL	GLCGAHSRNVLLIVA	D	DGGFESGVYNNNTAI	ATPHLDALSRHSLIF	RNAFTSVSSCSFSPRA	SLLTGLPQHONGMYG	90
Mutant	N	
C57BL/6	D	
WildType	LHQDVHFNFSFDKVQ	SLPLLLNQAGVRTGI		IGKKHVGPETVYPFD	FAFTEENSSVMQVGR	NITRIKQLVQKFLQT	QDDRPFLLVAFHDP	180
Mutant	
C57BL/6	
WildType	HRCGHSQPQYGTFCF	KFGNGESGMGYIPDW		TPQIYDPQDVMVPYF	VPDTPAARADLAAQY	TTIGRMDQGVGLVLQ	ELRGAGVLNDTLIIF	270
Mutant	
C57BL/6	
WildType	TSDNGIPFPPSGRTNL	YWPGTAEPLLVSSE		HPQRWGQVSDAYVSL	LDLTPTILDWFSIPY	PSYAIFGSKTIQLTG	RSLLPALAEPLWAT	360
Mutant	
C57BL/6	
WildType	VFSSQSHHEVTMSYP	MRSVYHQNFRLIHNL		SFKMFPIDQDFYVS	PTFQDLLNRTTGRP	TGWYKDLHRYYYRER	WELYDISRDPRETRN	450
Mutant	
C57BL/6	
WildType	LAADPDLAQVLEMLK	AQLVKWQWETHDPWV		CAPDGVLEEKLTPQC	RPLHNEL			502
Mutant			
C57BL/6G..				

Fig. 2. Amino acid sequence of wild type murine sulfamidase from the mainly CD1/129^{Svj} background aligned with the sequence from an affected mouse of the same background and a sequence from the C57BL/6 background (Costanzi *et al.*, 2000). The box identifies the point mutation (D31N). These sequences are deposited in GenBank and have the accession numbers AF304053 (wild type) and AF304054 (mutant).

gene. However, this was not the case as the parent ES cell line, WW6, and the mutant clone, WW6.186, were both shown to carry the wild type sequence at nucleotide 91 of the sulfamidase gene coding region (Figure 1). In addition, CD1 mice to which WW6.186 chimeras were mated in deriving the colony, are also wild type at this position (Figure 1). Therefore, the sulfamidase mutation was not preexisting in either the ES cells or in the mouse strain used to generate the colony. The mutation may have arisen from a rare heterozygous CD1 mouse, or from a mutation that occurred during the first few generations of interbreeding of WW6.186 × CD1 progeny that derived from the chimera.

Recombinant sulfamidase (D31N) is inactive in CHO cells

To assay the sulfamidase activity of wild type and mutant cDNAs, they were cloned into the pCDNA3.1 expression vector and transfected into CHO-K1 cells. Sulfamidase activity of independent clones was determined with a radiolabeled tetrasaccharide (Hopwood and Elliott, 1982). Sulfamidase expressed from mutant cDNAs had markedly reduced *N*-sulfatase activity (~3%) compared to wild type cDNAs (Table I). Therefore, the D31N missense mutation severely cripples sulfamidase activity. As would be predicted, correction of the A at nucleotide 91 to G by site-directed mutagenesis restored sulfamidase activity to wild type levels (Table I).

Discussion

The MPS III A mouse carries a novel sulfamidase mutation that has not previously been observed among humans with MPS III A (Figure 3). The mutation would be predicted to have

Table I. Specific activity of wild type and mutant murine sulfamidase

cDNA Transfected	Specific activity (pmol/min/mg protein)
Wild type	403, 549
Vector control	0.5, 2.1
Mutant (Clone 1)	21, 23
Mutant (Clone 2)	3.3, 8.7
Corrected mutant (Clone 1)	408, 508
Corrected mutant (Clone 2)	407, 586

Cell lysates from G418 resistant cultures of CHO-K1 cells expressing a cDNA encoding murine sulfamidase from wild type (mainly CD1/129^{Svj}), mutant (D31N) or mutant corrected by site-directed mutagenesis to wild type, were assayed using a radiolabeled tetrasaccharide. Duplicate results are shown.

a serious affect on enzyme function based on structural considerations. The aspartic acid residue D31 in combination with D32, is proposed to be involved in the coordination of a divalent metal ion needed for the catalytic event in the family of sulfatases that include sulfamidase (Bond *et al.*, 1997). Also, based on the crystal structure of *N*-acetylgalactosamine-4-sulfatase, two other amino acids D273 and N274 are likely to be involved in coordination of the divalent metal ion. Other amino acids proposed to be critical for catalysis in *N*-acetylgalactosamine-4-sulfatase based on the crystal structure (Bond *et al.*, 1997) are conserved, both in nature and relative position, in the primary sequence of human and mouse sulfamidase. Thus D53, D54, C91, P93, S94, R95, K145, H147, D300, N301, and K318 in *N*-acetylgalactosamine-4-sulfatase correspond

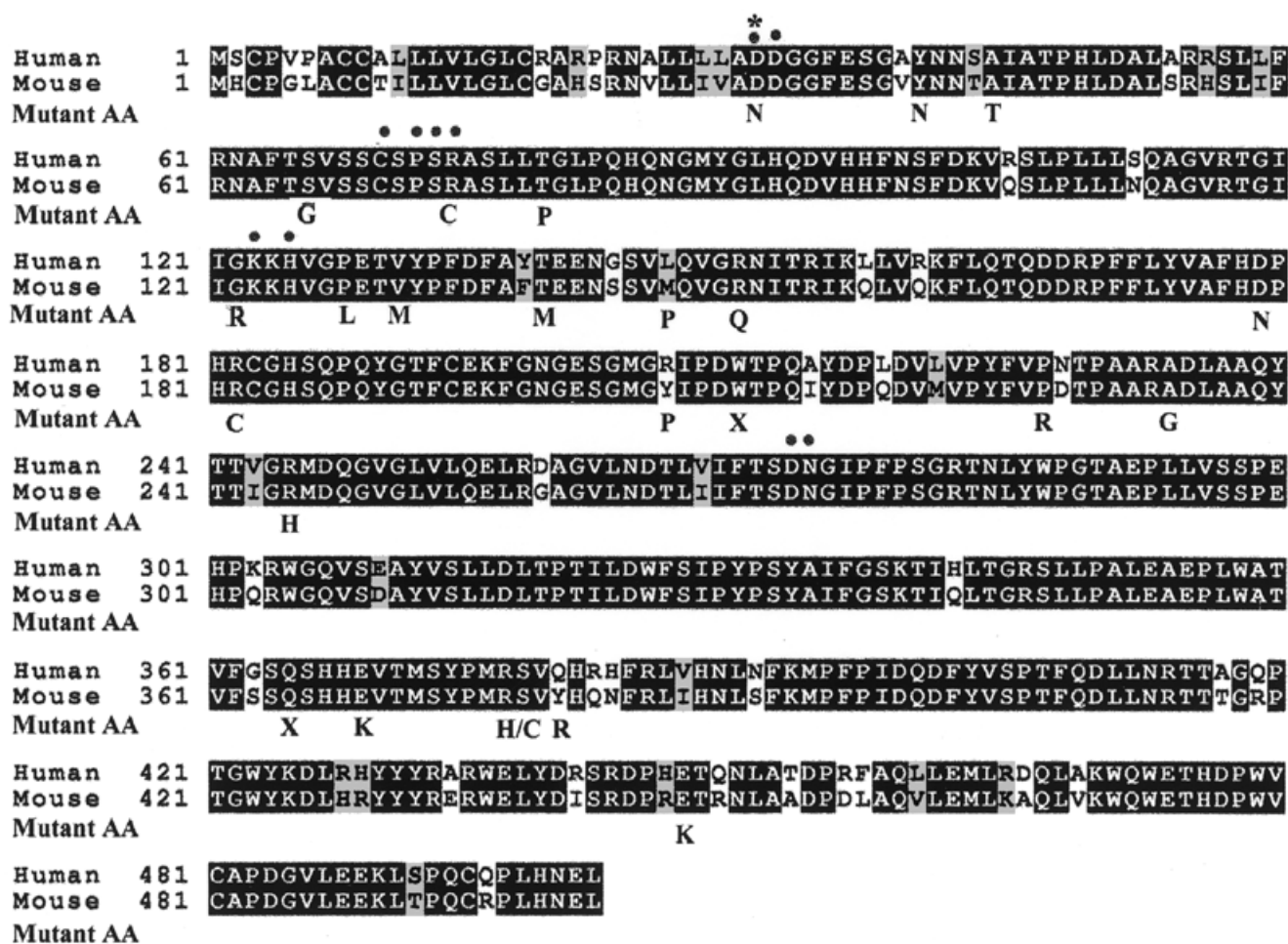


Fig. 3. Clustal W analysis of human and mouse (mainly CD1/129^{Svj} background) sulfamidase sequences with the location of the inactivating point mutation identified here in the mouse (*) and those identified previously in humans (Scott *et al.*, 1995; Blanch *et al.*, 1997; Bunge *et al.*, 1997; Weber *et al.*, 1997; Di Natale *et al.*, 1998; Montfort *et al.*, 1998; Esposito *et al.*, 2000) (Mutant AA). The amino acids proposed as critical for sulfatase activity in *N*-acetylgalactosamine-4-sulfatase (Bond *et al.*, 1997) are identified by a dot.

to D31, D32, C70, P72, S73, R74, K123, H125, D273, and N274 in human and mouse sulfamidase (Figure 3). The only exception is the predicted K at amino acid 303 that is conserved in human but not in mouse sulfamidase where it is a Q.

The MPS III A mouse is an excellent model for the human disease which arises, in many cases, from a point mutation in the sulfamidase gene coding region as summarized in Figure 3. Targeted sulfamidase gene deletion would not so closely mimic the human disease. It is clear from the spread of inactivating mutations almost throughout the molecule, that reductions in sulfamidase activity easily arise from single amino acid changes in the sequence. Mutant sulfamidase molecules might therefore be induced to refold into an active form if an appropriate chemical conformerone could be selected from a combinatorial library. This type of strategy was successful in improving the transport and maturation of α -galactosidase A in Fabry lymphoblasts (Fan *et al.*, 1999). The MPS III A mouse will be useful for evaluating pathogenic mechanisms arising from reduced sulfamidase activity and for testing a range of therapeutic strategies such as enzyme refolding or replacement, gene replacement and cellular transplantation therapies

for the human disease. The MPS III B mouse that lacks α -*N*-acetylglucosaminidase due to a targeted mutation and cannot degrade heparan sulfate, has a similar phenotype to the MPS III A mouse (Li *et al.*, 1999) and also provides an excellent mouse model for testing therapies for Sanfilippo syndrome.

Materials and methods

Mice

A colony of mice with the sulfamidase deficiency was established as described previously (Bhaumik *et al.*, 1999). These mice are of mixed genetic background including predominantly CD1 and 129^{Svj} with some C57BL/6 and SJL strain contributions. All studies using animals had the approval of the Institutional Care and Use Committee of the Albert Einstein College of Medicine.

Cloning mouse sulfamidase cDNA

Poly(A⁺) RNA was isolated from mouse liver using Trizol (Gibco BRL) and oligo-dT cellulose (Pharmacia). Oligo(dT)₁₂₋₁₈

(Gibco BRL) and superscript II RT enzyme (Gibco BRL) were used for reverse transcription. Gene specific primers based on the C57BL/6 mouse cDNA sequence (Costanzi *et al.*, 2000) were: 5' RESN (CGCTTGCGGCCGCGGAGCCGGAACCGC-TTACCCTGACC) from -33 to -10 from the ATG (+1); 3' RESH (GTCCCAAGCTTCTCCATGTCCAGGTGGGCTGGCAG) spanned nucleotide +1525 to +1548 in the 3' UTR just beyond the sulfamidase coding sequence. Expand PCR DNA polymerase (Boehringer Mannheim) was used to amplify the ~1.6 kb coding region. *NotI* and *HindIII* restriction sites in the primers (underlined) were used to clone cDNAs into pCDNA3.1(-) (Stratagene). PCR products and plasmid DNA prepared by the QIAGEN kit were sequenced. Mutant cDNA was corrected to wild type using site-directed mutagenesis (Quickchang™ kit, Stratagene). with primers PS330 (GCTACTGATAGTTGCG-GATGACGGAGGCTTTG) and PS331 (CAAAGCCTCCGT-CATCCGCAACTATCAGTAGC).

Genotyping MPS III A mice

Genomic DNA from mouse tail was amplified with primers PS339(5' GGTCTGTCTTCCTCAGCG) and PS340 (5' GATAAGGCTGTGGCGGGACAGGG) following 3 min at 94°C, 30 cycles of 45 sec at 94°C, 45 s at 60°C, 1 min at 72°C and 4 min final extension at 72°C. PCR products were purified with the QIAGEN PCR spin column and digested with *MspAII* (New England BioLabs) at 37°C for 2 h before electrophoresis on a 3.5% agarose gel. *MspAII* cuts between the G and C of the sequence CAGCGG.

Sulfamidase assay

To determine sulfamidase activity, cDNAs encoding wild type, mutant and corrected mutant sequences in pCDNA3.1(-) were transfected into CHO-K1 cells. G418 resistant transfectants were grown to confluence, lysates were prepared and assayed using a radiolabeled tetrasaccharide (glucosamine-*N*-sulfate-(1,4)-iduronic or glucuronic acid-1,4-glucosamine-*N*-sulphate-(1,4)-[³H]-iduronic, gluconic or anhydroidonic acid as described previously (Hopwood and Elliott, 1982; Bhaumik *et al.*, 1999).

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References

- Bhaumik, M., Muller, V.J., Rozaklis, I., Johnson, L., Dobrenis, K., Bhattacharya, R., Wurzelmann, S., Finamore, P., Hopwood, J.J., Walkley, S.U., and Stanley, P. (1999) A mouse model for mucopolysaccharidosis type III A (Sanfilippo syndrome). *Glycobiology*, **9**, 1389–1396.
- Blanch, L., Weber, B., Guo, X.H., Scott, H.S., and Hopwood, J.J. (1997) Molecular defects in Sanfilippo syndrome type A. *Hum. Mol. Genet.*, **6**, 787–791.
- Bond, C.S., Clements, P.R., Ashby, S.J., Collyer, C.A., Harrop, S.J., Hopwood, J.J., and Guss, J.M. (1997) Structure of a human lysosomal sulfatase. *Structure*, **5**, 277–289.
- Bunge, S., Ince, H., Steglich, C., Kleijer, W.J., Beck, M., Zaremba, J., van Diggelen, O.P., Weber, B., Hopwood, J.J., and Gal, A. (1997) Identification of 16 sulfamidase gene mutations including the common R74C in patients with mucopolysaccharidosis type IIIA (Sanfilippo A). *Hum. Mutat.*, **10**, 479–485.
- Costanzi, E., Beccari, T., Stinchi, S., Bibi, L., Hopwood, J.J., and Orlacchio, A. (2000) Gene encoding the mouse sulphamidase: cDNA cloning, structure and chromosomal mapping. *Mamm. Genome*, **11**, 436–439.
- Di Natale, P., Balzano, N., Esposito, S., and Villani, G.R. (1998) Identification of molecular defects in Italian Sanfilippo A patients including 13 novel mutations. *Hum. Mutat.*, **11**, 313–320.
- Esposito, S., Balzano, N., Daniele, A., Villani, G.R., Perkins, K., Weber, B., Hopwood, J.J., and Di Natale, P. (2000) Heparan N-sulfatase gene: two novel mutations and transient expression of 15 defects. *Biochim. Biophys. Acta*, **1501**, 1–11.
- Fan, J.Q., Ishii, S., Asano, N., and Suzuki, Y. (1999) Accelerated transport and maturation of lysosomal α -galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. *Nature Med.*, **5**, 112–115.
- Fischer, A., Carmichael, K.P., Munnell, J.F., Jhabvala, P., Thompson, J.N., Matalon, R., Jezyk, P.F., Wang, P., and Giger, U. (1998) Sulfamidase deficiency in a family of dachshunds: a canine model of mucopolysaccharidosis IIIA (Sanfilippo A). *Pediatr. Res.*, **44**, 74–82.
- Hopwood, J.J. and Elliott, H. (1982) Diagnosis of Sanfilippo type A syndrome by estimation of sulfamidase activity using a radiolabelled tetrasaccharide substrate. *Clin. Chim. Acta*, **123**, 241–250.
- Kresse, H. (1973) Mucopolysaccharidosis 3 A (Sanfilippo A disease): deficiency of a heparin sulfamidase in skin fibroblasts and leucocytes. *Biochem. Biophys. Res. Commun.*, **54**, 1111–1118.
- Li, H.H., Yu, W.H., Rozengurt, N., Zhao, H.Z., Lyons, K.M., Anagnostaras, S., Fanselow, M.S., Suzuki, K., Vanier, M.T., and Neufeld, E.F. (1999) Mouse model of Sanfilippo syndrome type B produced by targeted disruption of the gene encoding α -N-acetylglucosaminidase. *Proc. Natl. Acad. Sci. USA*, **96**, 14505–14510.
- Montfort, M., Vilageliu, L., Garcia-Giralt, N., Guidi, S., Coll, M.J., Chabas, A., and Grinberg, D. (1998) Mutation 1091delC is highly prevalent in Spanish Sanfilippo syndrome type A patients. *Hum. Mutat.*, **12**, 274–279.
- Neufeld, E.F. and Muenzer, J. (1995) The mucopolysaccharidoses. In Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D. (ed.), *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed. Mc Graw-Hill, New York, pp. 2465–2494.
- Poorthuis, B.J., Wevers, R.A., Kleijer, W.J., Groener, J.E., de Jong, J.G., van Weely, S., Niezen-Koning, K.E., and van Diggelen, O.P. (1999) The frequency of lysosomal storage diseases in The Netherlands. *Hum. Genet.*, **105**, 151–156.
- Scott, H.S., Blanch, L., Guo, X.H., Freeman, C., Orsborn, A., Baker, E., Sutherland, G.R., Morris, C.P., and Hopwood, J.J. (1995) Cloning of the sulphamidase gene and identification of mutations in Sanfilippo A syndrome. *Nature Genet.*, **11**, 465–467.
- Weber, B., Guo, X.H., Wraith, J.E., Cooper, A., Kleijer, W.J., Bunge, S., and Hopwood, J.J. (1997) Novel mutations in Sanfilippo A syndrome: implications for enzyme function. *Hum. Mol. Genet.*, **6**, 1573–1579.
- Whiteman, P. and Young, E. (1977) The laboratory diagnosis of Sanfilippo disease. *Clin. Chim. Acta*, **76**, 139–147.