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Molecular Cloning of the Acid Sphingomyelinase of the Mouse and the Organization and Complete Nucleotide Sequence of the Gene

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Summary: The cDNA and complete gene encoding mouse acid sphingomyelinase (ASM) has been isolated using a homology screening approach. Comparison with the human sequence shows 81% sequence identity at cDNA level and 82% at the protein level. Homology is markedly reduced in the N-terminal region, especially in the presumed signal peptide. The six-exon gene structure is similar to the human one, except for the position of *Alu*1 elements. The alternatively used splice site 40 bp downstream of the human exon 2 is not conserved in the mouse gene and accordingly no such alternative splicing was found in mouse.

Klonierung der sauren Sphingomyelinase der Maus, ihre Genorganisation und die vollständige Nucleotidsequenz des Gens

Zusammenfassung: Die cDNA und das vollständige, die saure Sphingomyelinase (ASM) kodierende Gen der Maus wurden mit der Homologie-Screening-Methode isoliert. Der Vergleich der ASM-kodierenden Sequenzen von Maus und Mensch ergab 81% Identität auf der cDNA-Ebene und 82% auf der Proteinebene. Die Homologie ist im N-terminalen Bereich, besonders im vermutlichen Signalpeptid, deutlich herabgesetzt. Die sechs Exons aufweisende Genstruktur ist der des menschlichen Gens ähnlich, abgesehen von der Position der *Alu*1-Elemente. Die alternative Splice-Stelle, 40 Basenpaare 3′ gelegen vom Exon 2 im menschlichen Gen, ist nicht konserviert. Entsprechend konnte kein alternatives Splicing in Maus-mRNA nachgewiesen werden.

Key terms: Human and mous acid sphingomyelinase/cDNA/genomic DNA/gene nucleotide sequence/homologies.

Acid sphingomyelinase (ASM) (sphingomyelin phosphodiesterase, EC 3.1.4.12) catalyses the cleavage of sphingomyelin to ceramide and phosphocholine. The enzyme activity is represented in all cells as an acid lysosomal hydrolase. Deficient ASM activity is shown to be responsible for Niemann-Pick disease

Enzymes:
Sphingomyelin phosphodiesterase, sphingomyelin cholinephosphohydrolase (EC 3.1.4.12);
Polynucleotide 5′-hydroxyl-kinase, ATP:5′-dephosphopolynucleotide 5′-phosphotransferase (EC 2.7.1.78), in this paper named polynucleotide kinase;
RNA-directed DNA polymerase, deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (RNA-directed) (EC 2.7.7.49), also named reverse transcriptase.

Abbreviations:
ASM, acid sphingomyelinase; NPD, Niemann-Pick disease; PCR, polymerase chain reaction; SSC, 0.15M sodium chloride, 0.015M sodium citrate; SDS, sodium dodecyl sulfate.

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-781  ggatcctcttccggcctccagcaggcaccacgcacgtcgtagcacacatacaaaatacccc  -722
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-661  ccttaagtgattcaaggctgctgtaatggtggcagagcataggaaatcgagggaggacc  -602
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 -61  agccagggcttcggcctgtggggcctggaggggcaagccagtcctgctacgagcctggc  -2

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       MetProHisHisArgAlaSerSerGlyGlnAspHisLeuArgAlaGlyTrpGluGlnAr

  60  ACTGGAGAGGTCCTTACCGGCACCCAGAGTGGGACTCCTTTGGATGGGGCTGGGCTTGGC  119
      gLeuGluArgSerLeuProAlaProArgValGlyLeuLeuTrpMetGlyLeuGlyLeuAl

 120  GCTGGTTCTGGCTCTGTTTGACTCCACGGTTCTTTGGGTTCCTGCCAGAGCTTATCCTCT  179
      aLeuValLeuAlaLeuPheAspSerThrValLeuTrpValProAlaArgAlaTyrProLe

 180  TCCTTCTGAAGGCCATTCTGTCAAATTCAGTGCCATAGCGCCGCCGCTCCAGAGTGCCTT  239
      uProSerGluGlyHisSerValLysPheSerAlaIleAlaProProLeuGlnSerAlaPh

 240  TGGGTGGCAGAACCTCACTTGCCCCGCCTGCAAAGTCTTATTCACTGCTCTCAACCATGG  299
      eGlyTrpGlnAsnLeuThrCysProAlaCysLysValLeuPheThrAlaLeuAsnHisGl

 300  GCTGAAGgtgagtgcgtgaagggctgttgtggaatgctgggggcaggatgaagtgcactg  359
      yLeuLys

 360  gtatgcccgggggagggggcaatatatttctggtagagggtgcttcctatgcatccatgt  419
 420  taccactaccccactgtgacctttataaagttagggagagtaaagtcagctggctaattg  479
 480  tgggcttgaaatggtagcacaccctgagctagagtaggtagctagagggcctggtgctgaa  539
 540  ctgctgggcagtgtgtgggagatactgatgtctctttatcatggtgacagagttatccta  599
 600  gcctccgatttagacatggagcctgtggatggtggccagggttcacttgttctgtctctg  659

 660  acctaacttcattggttccttccaccacagAAGGAGCCCAATGTGGCACGGGTAGGCTCT  719
                                        LysGluProAsnValAlaArgValGlySer

 720  GTGGCCATCAAGATATGCAAGATGCTGAACATAGCACCACTAGATGTGTGCCAGTCAGCC  779
      ValAlaIleLysIleCysLysMetLeuAsnIleAlaProLeuAspValCysGlnSerAla

 780  GTCCATCTCTTTGAGGACGATGTGGTGGAGGTGTGGACACGTTCAGTTCTGAGCCCATCA  839
      ValHisLeuPheGluAspAspValValGluValTrpThrArgSerValLeuSerProSer

 840  GAGGCTTGTGGCTTGCTTCTGGGCTCCTCTTGTGGACACTGGGACATCTTTTCGACTTGG  899
      GluAlaCysGlyLeuLeuLeuGlySerSerCysGlyHisTrpAspIlePheSerThrTrp

 900  AACATCTCTTTGCCATCAGTGCCGAAGCCACCCCCAAAGCCACCGAGCCCACCAGCCCCC  959
      AsnIleSerLeuProSerValProLysProProProLysProProSerProProAlaPro

 960  GGTGCCCCTGTCAGCCGTGTCCTCTTCCTTACTGATCTACACTGGGACCATGAGTACCTG  1019
      GlyAlaProValSerArgValLeuPheLeuThrAspLeuHisTrpAspHisGluTyrLeu

1020  GAGGGCACAGACCCTTACTGTGCAGATCCACTTTCTGGCCGCCGGGGGTCCGGATGGCCA  1079
      GluGlyThrAspProTyrCysAlaAspProLeuSerGlyArgArgGlySerGlyTrpPro

1080  CCCAACTCCCAGAAGGGGGCTGGGTTCTGGGGCGAGTACAGCAAGTGTGACCTGCCCTTG  1139
      ProAsnSerGlnLysGlyAlaGlyPheTrpGlyGluTyrSerLysCysAspLeuProLeu

1140  CGAACGCTAGAAAGCCTGTTGAAAGGACTGGGCCCTGCCGGCCCTTTTGAAATGGTGTAC  1199
      ArgThrLeuGluSerLeuLeuLysGlyLeuGlyProAlaGlyProPheGluMetValTyr

1200  TGGACGGGAGATATCCCTGCCCATGATGTCTGGCAACAGTCTCGCCAAGATCAGCTGAGG  1259
      TrpThrGlyAspIleProAlaHisAspValTrpGlnGlnSerArgGlnAspGlnLeuArg

1260  GCCCTGACCACCATCACAGACCTCGTGAGGAAGTTCTTGGGGCCAGTGCCAGTGTACCCT  1319
      AlaLeuThrThrIleThrAspLeuValArgLysPheLeuGlyProValProValTyrPro

1320  GCTGTGGGCAACCATGAGAGCACTCCTGTCAATGGCTTCCCTCCTCCCTTCATAAAGGGA  1379
      AlaValGlyAsnHisGluSerThrProValAsnGlyPheProProProPheIleLysGly

1380  AACCAATCTTCACAATGGCTCTATGAAGCCATGGCCAAGGCATGGGAACCCTGGTTACCA  1439
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1440  GCTGATGCCCTTCACACCCTAAGgtacttactgtcggtggaaacccaggaaggaagaaga  1499
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1500  aagatggatgagggagaaaggaggagggaactgggtagactactccagcaggtgtcctca  1559
1560  gcactctctactgccctccctaacctgaccctgccttccctttaattatctccagccacc  1619
1620  ctcttttcagagctagcagtacatccccactccctagcttgtgtctttgcctacagtagc  1679
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1800  gtcctgaattcatgaagctctcaaccctctgaggcttctttactgtcagaggagccctat  1859
1860  aattactgctgttggtcttcagctagggtgggagctcctgggggtgggagatgacatcat  1919
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1920  gtgtacctctgcctgatgtgcaggactattgacatctcggttacttttgtttcagAATTG   1979
                                                            gIleG

1980  GGGGCTTCTATGCCCTCACCCCACGCCCTGGCCTCCGCCTCATCTCTCTCAATATGAATT   2039
      lyGlyPheTyrAlaLeuThrProArgProGlyLeuArgLeuIleSerLeuAsnMetAsnP

2040  TTTGTCCCCGTGAGAACTTTTGGCTCTTGATCAACTCCACAGATCCTGCTGGACAACTCC   2099
      heCysProArgGluAsnPheTrpLeuLeuIleAsnSerThrAspProAlaGlyGlnLeuG

2100  AGTGGCTGGTGGAGGAGCTTCAGGCTGCTGAGAATCGAGGAGACAAAgtaaggggatgcg   2159
      lnTrpLeuValGluGluLeuGlnAlaAlaGluAsnArgGlyAspLys

2160  ttatgaaccgggtggagaaggtgctgttgggggctgaggagaagcaggctgctgtgtgtg   2219
2220  gggctgagatctgtgaagaggggagtgtcgcttcacttcctagaactactgtcttccctg   2279
2280  ggtccatgttcaccttcctgtgtccttcgtatactcaatcttctgtcactctacccaagg   2339

2340  ctgcctggattcctcaatgctctgactgcccttaattctcccttcagGTGCATATAATTG   2399
                                                      ValHisIleIleG

2400  GCCACATCCCTCCAGGACATTGTCTTAAGAGCTGGAGCTGGAATTATTACAAAATCATAG   2459
      lyHisIleProProGlyHisCysLeuLysSerTrpSerTrpAsnTyrTyrLysIleIleA

2460  CCAGgtaagggatagagggcttgggtgtggagactgaagggttaaaatactctcaacagt   2519
      laAr

2520  caggcagtggtagcacatgcctttaatcgaagcactcttgagttcaaggccagcctggtc   2579
2580  tacaaagtgagttctaggacagccagggctgttaacacagagaaatcctttctctggaga   2639
2640  aaaaaaaaaagaaagggagggaccgagggaggaaggagagagcaattccctcaagcacag   2699
2700  agtctcacaatctggaggagctactttgcccattgcagaacgaccaccctaactcctcag   2759

2760  gtctacccaccctcatttccacttccttacagccttctatcactgtgtctctgggcagGT   2819
                                                                gT

2820  ATGAAAACACTCTGGCCGGTCAGTTCTTTGGCCACACTCACGTGGATGAGTTTGAGATCT   2879
      yrGluAsnThrLeuAlaGlyGlnPhePheGlyHisThrHisValAspGluPheGluIleP

2880  TCTATGATGAGGAAACTCTGAGCCGACCACTAGCTGTAGCCTTCCTGGCGCCCAGTGCTA   2939
      heTyrAspGluGluThrLeuSerArgProLeuAlaValAlaPheLeuAlaProSerAlaT

2940  CAACCTTTATCAACCTTAACCCTGgtgagtaaggcagaagggagcattcctcatcctgag   2999
      hrThrPheIleAsnLeuAsnProG

3000  gctggtgtgacagaaagggatggaagccaaagcaggtcctccctaaagttcctttgttcc   3059

3060  cttccttaccactcaaccctcccttctcgcagGCTACCGAGTTTACCAAATAGATGGAAA   3119
                                          lyTyrArgValTyrGlnIleAspGlyAs

3120  CTACCCCGGAAGCTCTCATGTGGTCCTGGACCACGAGACCTACATCTTGAATCTGACCCA   3179
      nTyrProGlySerSerHisValValLeuAspHisGluThrTyrIleLeuAsnLeuThrGl

3180  GGCCAACGCAGCAGGAGGCACACCAAGCTGGAAGCGCCTCTACAGGGCTCGAGAAACCTA   3239
      nAlaAsnAlaAlaGlyGlyThrProSerTrpLysArgLeuTyrArgAlaArgGluThrTy

3240  TGGACTTCCAGATGCGATGCCTGCCTCCTGGCACAACCTGGTCTACCGCATGAGGGATGA   3299
      rGlyLeuProAspAlaMetProAlaSerTrpHisAsnLeuValTyrArgMetArgAspAs

3300  TGAGCAACTCTTCCAGACCTTCTGGTTTCTCTACCATAAGGGCCACCCACCTTCAGAGCC   3359
      pGluGlnLeuPheGlnThrPheTrpPheLeuTyrHisLysGlyHisProProSerGluPr

3360  CTGCGGCACACCCTGCCGCCTGGCCACTCTGTGTGCCCAGCTCTCAGCCCGAGCAGACAG   3419
      cCysGlyThrProCysArgLeuAlaThrLeuCysAlaGlnLeuSerAlaArgAlaAspSe

3420  CCCTGCTTTGTGTCGCCACTTGATGCCCAATGGGAGCCTCCCAGATGCTAATAGGTTGTG   3479
      rProAlaLeuCysArgHisLeuMetProAsnGlySerLeuProAspAlaAsnArgLeuTr

3480  GTCACGCCCCCTGTTGTGCTAGtattccatgttccagactggggaaagttcatgtatcag   3539
      pSerArgProLeuLeuCys *

3540  aaaagggatacattcccgagtgctgcttatctacctgaggcaaaagcttcgggggaagga   3599
3600  gccagccaggccagggaaacaagccgcctcatgagagcttgtttggttacactggaggag   3659
3660  gttttggctgtcctacttatgcccaggatctggattacccctggggctgatgtctgacct   3719
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3780  gctaatggacctctgaaccctgtactgttacctgatcctgcaaggctgttaaaataaaga   3839
3840  gacacacttcagactccaccgttcctatttcttcttcccaaacaggcagggcaaagggag   3899
3900  gttggtagcatgaccctgactgaagagttgcatgagcacaggaagacacacagcagcgca   3959
3960  gacagactttattagtgatatcagtacagcggagg  3994
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Fig. 1. Complete nucleotide sequence of the mouse ASM gene with translated exon sequences.

(NPD) of a neuronopathic, type A, and a visceral, nonneuronopathic, type B, form. Although in both storage disease types similar residual ASM activity (0–10% of normal level) can be measured, they differ in their phenotype and the severity of the symptoms. The neuropathological symptoms of NPD-A lead to death within the first three years of age, whereas NPD-B patients show only visceral symptoms and often have a normal lifespan[1]. Recent cloning and sequencing of human ASM[2,3] opened up the possibility to analyse the molecular basis of these lipid storage disorders. Subsequently nine different mutations[4–8] have been found in the ASM gene causing NPD type A or type B. Despite of this progress the different development of the neuronopathic (NPD-A) and visceral form (NPD-B) caused by mutations of the sphingomyelinase gene is not understood in molecular terms. To answer this question and to examine the possibility of a gene therapy, a well characterized animal model would be helpful. Existing animal models[9–12] are insufficiently characterized on the genetic level. For this reason we are developing a NPD-mouse model based on transgene technology. Such transgenic mice would offer the possibility to prove ASM-point mutations as sole basis of the NPD. Here we report as a first step the mouse acid sphingomyelinase cDNA and the complete nucleotide sequence of the corresponding gene.

Table 1. List of used oligonucleotides.

Primers for PCR generating the screening fragment:		
Oh1	(cctgccgtgtgccaatccattgtccacctc)	Exon 2, human, sense
Oh2	(gaagctattgacaggtatgctttcatggtt)	Exon 2, human, antisense
Primers for PCR amplification of mouse ASM-cDNA:		
Om3	(ctggttaccagctgatgcccttcacaccct)	Exon 2, mouse, sense
Om4	(ggcccttatggtagagaaaccagaaggtct)	Exon 6, mouse, antisense
Oligonucleotides used for Southern blot hybridization analysis:		
Om5	(ttactgtcggtggaaacccaggaaggaaga)	alternative splice region, mouse, sense
Om6	(ctctctcaatatgaattt)	Exon 3, mouse, sense
Om7	(gcatataattggccacat)	Exon 4, mouse, sense
Om8	(cacactcacgtggatgag)	Exon 5, mouse, sense
Oligonucleotides used for primer extension reaction:		
Om9	(ctggccggatgatgctctgtggtggggcat)	Exon 1, mouse, antisense
Oh10	(tcctacacggggctggttcgtctgtcccgt)	Exon 1, human, antisense

Methods and Materials

Isolation of cDNA and genomic clones

Human genomic (Clontech, HL1006d, λ-EMBL3), mouse genomic (Clontech, ML1040j, λ-EMBL3-Sp6/T7) and mouse liver cDNA (Clontech, ML 1035b, λgt11) libraries were screened, using PCR amplified genomic fragments with primers (Table 1: Oh1, Oh2) derived from the human ASM cDNA sequence. Libraries were screened by standard procedures[13] on cellulose nitrate filters (Schleicher & Schuell, BA-85). Filters were hybridized with the radiolabeled PCR fragment and stringently washed with 0.2 × SSC, 0.1% SDS at 55 °C. Positive plaques were identified by autoradiography and purified.

DNA sequencing

The cDNA and the genomic inserts of the ASM clones were subcloned into the plasmid pUC19 and sequenced using the standard method for double-strand sequencing[14,15] (Pharmacia, T7-Sequenase Kit). Sequence analysis and comparisons were carried out using the GCG program package.

Southern blot hybridization analysis

The synthetic oligonucleotides (Om5–8, listed in Table 1) derived from the ASM nucleotide sequences were labeled with [^{32}P]ATP in a polynucleotide kinase reaction. They were used for the Southern blot hybridization (Dupont, gene screen plus) of the PCR fragments (with primers Om3 and Om4) separated by agarose gel electrophoresis and of dot-blotted genomic fragment. cDNA used as the PCR template was generated by standard reverse transcriptase reaction (superscript reverse transcriptase, BRL) with mouse brain poly(A)$^+$ RNA.

Primer extension

The primer extension reaction was carried out following established methods[17] using the superscript reverse transcriptase (BRL) with the primers Om9 and Oh10 (see Table 1).

Results and Discussion

The complete mouse ASM gene (Fig. 1) was isolated and subcloned in pUC19 as a 4.8-kb *Bam*HI fragment, starting 780 bp upstream the start codon and ending 160 bp downstream the polyadenylation signal. The human ASM gene was isolated and subcloned in pUC19 as a 3.2 kb *Bam*HI fragment (5′ domain) and a 1.9 kb *Bam*HI-*Eco*RV fragment (3′ domain), starting 520 bp upstream the start codon and

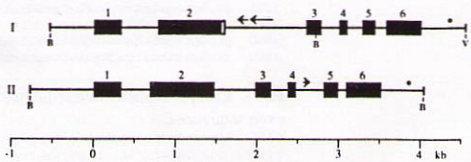
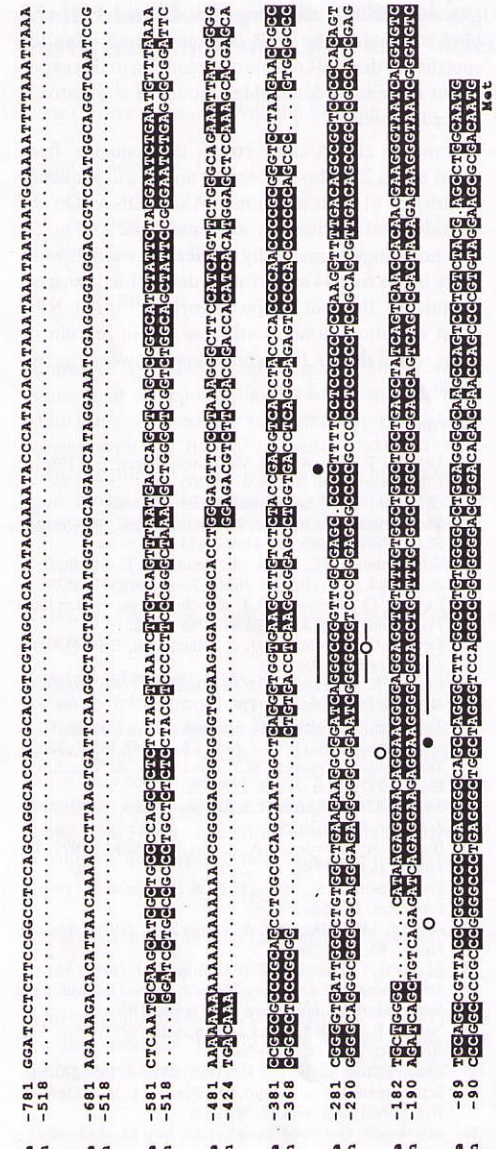


Fig. 2. Gene structure of human ASM (I) and mouse ASM (II). Arrow: *Alu*1 element; open box: alternative splice region in the human gene; open circle: polyadenylation signal; B: *Bam*HI restriction site; V: *Eco*RV restriction site.


Mouse -781 GGATCCTCTTCCGGCCTCCAGCAGGCACCACGCACGTGTAGCACACATACAAAATACCCATACATAAATAAATAATAATGCAAAATTTAAATTAAA
Human -518
Mouse -681 AGAAAGACACATTAACAAAACCTTAAGTGATTCAAGGCTGCTGTAATGGTGGCAGAGCATAGGAATTCGAGGGGAGGACCGTCCATGGCAGGTCAATCCG
Human -518
Mouse -581 CTCAATGGAAGATCGTGCCCCAGCCCTCTAGTAATTTTCCAGTTTAATTGACCAGCCTGAGCCGGGGCATCTATTCTAGGAATCTGATTGTTTAAA
Human -518 GCATCTGCCTGCCTACCTCCCCTCCCGGCTAAAGCTCGGCGCGGCGGCATCTGGCATCTATCCGGAATCTGACCCCGGATTC
Mouse -481 AAAAAAAAAAAAAAAAAAGCGGGGGGTGGAAGAGAGGGCACCTGGAGTGCCCAAGGCCTTCCAGTCTCGGCGCAAATTTCGGGG
Human -424 TGCAAA GGAGACCTCTCCACCACCACATCACAGGACTCTCCAACGCAGCAGCA
Mouse -381 CCGCGCGCGCGCAGCCCTCGCGCAGCAATGGCTGAGCTTGTCTCACCGGAGCCCCCGCGCGCGCTAAGAGCGCC
Human -368 CGGCCTCCCCCGG CTTGACCTCAGGCGCAGCCTCGTGACCTCAGGAGTCCCACCCCGGAGCCCC GTGGCCC
Mouse -281 CGGGTGATTGGCCTGTGGCCTGAAGAAGGCGATCAGGCGGGTTCCGCGAGCCCCCCCTTTTCCTCCGCGCCCCAAGCTTTTGAGAGCGCCTTCCAGAGT
Human -290 GGGCCAGGCCGGGGCCAGAGGGCCCCAATCCGGGCGGTCCCCGCGAGCCCCCCCCTCCCCCCGCACGCCCTTTGACAGCGCCCCCGCCCCGAG
Mouse -182 TGCGG ...CTAAGAGGAAGAGGAGCGCAGGAGCTGTTTTCTCGGCGCGCGCGC

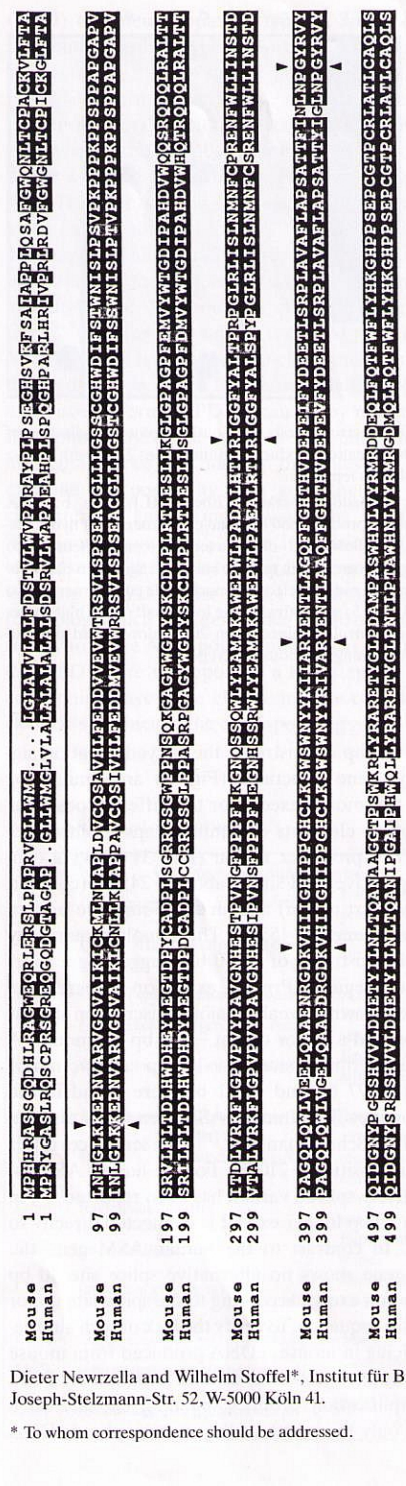


Fig. 5. Comparison of the ASM amino-acid sequence of mouse and human.
Lines mark the signal peptide according to the van Heijne algorithm; arrow heads mark the exon boundaries.

on gel electrophoresis (Fig. 4). Southern blotting and subsequent hybridisation with a radiolabeled oligonucleotide complementary to the alternatively spliced region did not mark any PCR product. Radiolabeled oligonucleotides complementary to exon 3, 4, or 5 bind strongly to the PCR fragment, confirming the specificity of the PCR reaction. According to this experiment there is no detectable amount of alternatively spliced transcript.

The mouse cDNA clone covers the sequence from −118 bp to 2221 bp and shows an overall sequence identity of 81% to the human ASM cDNA. On the peptide level the identity amounts to 82% (Fig. 5). The homology is markedly reduced in exon 1, especially in the first 44 amino acids determined as signal peptide by the van Heijne algorithm[17]. All NPD point mutations found until now are in protein regions which show a 100% homology between the two species.

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The sequence data reported in this paper have been submitted to the GenBank Database Library under the accession numbers X63600 (ASM human gene), Z14252 (ASM mouse cDNA) and Z14132 (ASM mouse gene).

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