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Complex Repetitive Arrangements of Gene Sequence in the Candidate Region of the Spinal Muscular Atrophy Gene in 5q13

A. M. Theodosiou,[1] K. E. Morrison,[2,3] A. M. Nesbit,[1] R. J. Daniels,[2] L. Campbell,[2] M. J. Francis,[2] Z. Christodoulou,[2] and K. E. Davies[1,2]

[1]MRC Clinical Sciences Centre, Royal Postgraduate Medical School, Hammersmith Hospital, London; and [2]Molecular Genetics Group and [3]Neurosciences Group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford

Summary

Childhood-onset proximal spinal muscular atrophy (SMA) is a heritable neurological disorder, which has been mapped by genetic linkage analysis to chromosome 5q13, in the interval between markers D5S435 and D5S557. Here, we present gene sequences that have been isolated from this interval, several of which show sequence homologies to exons of β-glucuronidase. These gene sequences are repeated several times across the candidate region and are also present on chromosome 5p. The arrangement of these repetitive gene motifs is polymorphic between individuals. The high degree of variability observed may have some influence on the expression of the genes in the region. Since SMA is not inherited as a classical autosomal recessive disease, novel genomic rearrangements arising from aberrant recombination events between the complex repeats may be associated with the phenotype observed.

Introduction

Childhood-onset proximal spinal muscular atrophy (SMA) affects at least 1 in 20,000 births and is a significant source of childhood morbidity and mortality (Bundey 1985). The disease is associated pathologically with degeneration of alpha motor neurons; the precise biochemical defect remains to be determined. SMA is clinically heterogeneous, with patients being classified into three groups, according to the age at presentation and clinical severity (Munsat 1991). The disease is inherited as an autosomal recessive, but the segregation ratio in affected sibships is often less than the expected .25, particularly when the disease has an onset after the age of 6 mo (Bundey and Lovelace 1975; Hausmanova-Petrusewicz et al. 1985).

The mutation responsible for the three types of SMA has previously been localized to human chromosome 5q13, in the interval between the loci D5S435 and D5S557 (Brzustowicz et al. 1990; Gilliam et al. 1990; Melki et al. 1990*a*, 1990*b*; Sheth et al. 1991; Francis et al. 1993; Soares et al. 1993). Analysis of key recombinant families by several groups maps the mutation to this general region, indicating that significant genetic heterogeneity does not exist for this disorder (Morrison et al. 1992, 1993*b*; Kleyn et al. 1993; Melki et al. 1993; Wirth et al. 1994). Linkage disequilibrium has been reported between several of these genetic markers and the disease in the French Canadian population and, more recently, with marker CATT in cohorts of mixed-European descent (Burghes et al. 1994*b*; Simnard et al. 1994). De novo and inherited deletions have also been identified in a small proportion of type I SMA individuals with certain of the microsatellite markers that map within the interval between D5S435 and D5S557 (Melki et al. 1994).

Two independent YAC contigs have been constructed across the candidate region (Francis et al. 1993; Kleyn et al. 1993). The data from such YAC mapping are consistent with the order of loci determined from radiation hybrid mapping (Thompson et al. 1993). Preliminary analysis of the YACs indicated the presence of a high density of chromosome 5–specific repeats in this region. Many of the probes generated from bacteriophage or cosmid subclones of these YACs map to both 5q and 5p, by FISH (Francis et al. 1993).

In this paper, we describe the isolation of gene sequences that map within the highly repetitive candidate region. These closely related gene sequences show stretches of homology to several exons of the β-glucuronidase gene, which itself maps to human chromosome 7. The possible relevance of such repetitive motifs to the genetic mutations underlying SMA is discussed.

Families and Methods

Families

More than 200 SMA families, most with at least two affected individuals, were genotyped with proximal 5q markers. The diagnostic criteria adhered to were as suggested by the International SMA Consortium, as described elsewhere (Morrison et al. 1992).

DNA Preparation and Southern and PCR Analysis

DNA was prepared from peripheral blood lymphocytes or Epstein-Barr virus–transformed cell lines by standard

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Address for correspondence and reprints: Dr. Kay E. Davies, Molecular Genetics Group, Institute for Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom.

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methods. After digestion with the appropriate restriction enzyme, samples were electrophoresed on 0.8% agarose and were transferred to nylon membranes (Hybond N; Amersham). Marker probes were labeled by standard random priming, hybridized in Church and Gilbert buffer, and were visualized by autoradiography at −70°C for 2 d. The probes used for Southern blot analysis included pM4 (locus D5S6), pJK53 (locus D5S112), and p105-153 Ra (D5S39), as detailed elsewhere (Morrison et al. 1992). Microsatellite markers were genotyped using PCR conditions as described elsewhere: EF1/2a and EF13/14 (locus D5S125) (Morrison et al. 1993*b*); LAS96 (locus D5S681) (Morrison et al. 1993*a*); cVS19 (locus D5S435) (Soares et al. 1993); 2AE9.1 (locus D5S557) (Francis et al. 1993); 38.3 (locus D5S610) (Francis et al. 1993); RB104 (locus MAP1B) (Soares et al. 1993); JK53CA1/2 (locus D5S112) (Morrison et al. 1992); and 153-6741GT (locus D5S39) and YNCT (locus D5S127) (Daniels et al. 1992).

Isolation and Manipulation of YAC Clones

Yeast clones containing YACs were isolated by screening several YAC libraries, as previously described, using either PCR-based techniques or hybridization probes generated from YAC ends (Francis et al. 1993). Agarose plugs containing DNA from the YAC host were prepared by standard methods (Anand et al. 1989). For restriction-enzyme analysis, the agarose plugs were washed three times in 1 × Tris-EDTA (TE) at 50°C and equilibrated in the appropriate restriction-enzyme buffer on ice for 30 min prior to digestion. The YACs were digested with various enzymes overnight, and the products were fractionated by pulsed-field gel electrophoresis (LKB) in 0.5 × Tris-borate-EDTA buffer, 1.2% agarose (Sigma type II), with switch times dependent on the size separation required. Gels were depurinated using 0.25 M HCl for 10 min prior to blotting and hybridization.

Construction of Bacteriophage and Cosmid Libraries from YACs

Phage libraries were constructed in lambda GEM-12 genomic cloning vector (Promega), as described elsewhere (Sambrook et al. 1989). End clones were isolated by screening the libraries with pBR322-derived sequences specific to the YAC left and right arms. Relevant YACs were also subcloned into cosmids, using partial *Mbo*I digestion. The vector used was SuperCos-1 (Stratagene), and the procedure was modified from the manufacturer's recommendations for cloning total human genomic DNA. Transformants were screened with Cot-1 DNA. The human clones thus identified were picked into broth, and aliquots were stored in glycerol in microtiter plates.

HinfI Fingerprinting of Cosmids

Two hundred nanograms of each cosmid were digested at 37°C for 1 h with 5 U of *Hin*fI (Boehringer Mannheim) in a total reaction volume of 6 μl in microtiter wells. Two microliters of a master mix of labeling reaction containing 5 μl of buffer (10 mM Tris pH 7.4, 10 mM $MgCl_2$, 6 mM DTT); 5 μl of 1 mM dATP, dGTP, and dTTP; 38.5 μl of TE; 2 μl of Klenow at 5 U/μl; and 1 μl of [α^{32}P]-dCTP at >3,000 Ci/mmol (Amersham) were added to each digestion well, and the reaction was left at room temperature for 10 min. The reaction was completed by the addition of formamide loading buffer with dyes (95% formamide, 20 mM EDTA, 0.05% each of xylene cyanol and bromophenol blue). The microtiter plates were heated to 85°C for 10 min and were quenched on ice prior to loading of 3 μl of each sample onto 4% denaturing polyacrylamide gels. Electrophoresis was for 2 h. The gels were fixed and dried and exposed to X-ray film at −70°C for 2–3 h.

cDNA Library Screening

A total of 1.4×10^5 recombinant clones of a human fetal (25 wk) brain lambda gt10 library (Genentech) (Cousens et al. 1986) were screened with Ox5.5 positive subclones of an Ox5.5 positive cosmid derived from YAC 17F5. Approximately 3×10^4 clones of an adult human brain lambda gt10 library (again from Genentech) were also screened. Positive phage subclones were cloned into plasmid and were sequenced using standard methods (Sequenase; USB).

FISH Analysis of cDNA Clones

Metaphase chromosome spreads were prepared from phytohemagglutinin-stimulated whole blood by standard techniques. Insert DNA from each cDNA clone was labeled with biotin-16-dUTP (Boehringer Mannheim) by nick translation. The labeled probe was coprecipitated with sonicated salmon sperm DNA (Sigma) and RNA (BRL) to give a final concentration, in hybridization mix, of 2 μg/μl. A probe concentration of 5 ng/μl was used, with 300 ng of human Cot-1 competitor DNA (BRL)/μl. Hybridization and detection procedures were as described elsewhere (Francis et al. 1993).

Results

Mapping of SMA to the Interval between D5S435 and D5S557

Two hundred forty SMA families (principally from the United Kingdom and Finland) were genotyped with markers mapping in 5q11.2–13.3. All families studied gave genotype data consistent with linkage to markers in this region, providing no evidence of genetic heterogeneity. Two families were identified with recombination events that allow SMA to be mapped distal to locus D5S435, in keeping with other studies (Soares et al. 1993; Burghes et al. 1994*a*; Clermont et al. 1994). Elsewhere we have identified 2AE9.1 (locus D5S557) as the distal flanking marker for SMA (Francis et al. 1993). No further recombination events with this marker were identified in the expanded patient database analyzed in the present study.

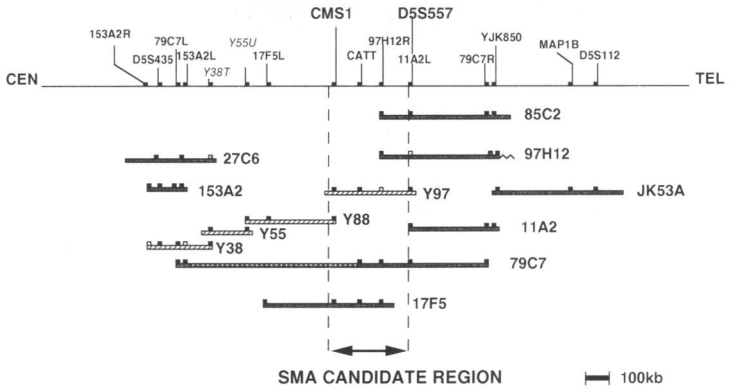


Figure 1 YAC contig spanning the SMA candidate region in 5q13. YAC libraries screened include the ICRF library (48,XXXX), the CEPH library (46,X,Y), and the ICI library (48,XXXX) (for details, see Francis et al. 1993; Kleyn et al. 1993). YAC clones denoted as blackened (▬) are as detailed by Francis et al. (1993). YAC 79C7 is deleted for the portion shown as dotted (▬). YACs denoted as hatched (▨) are as detailed by Kleyn et al. (1993). STSs present in the YACs are shown as blackened squares (■); and those shown as unblackened squares (□) were not tested. STS sequences are as reported by Francis et al. (1993), Kleyn et al. (1993), and Burghes et al. (1994*b*).

Isolation and Sequencing of cDNAs

A schematic representation of the YACs covering the SMA candidate region is shown in figure 1 (data are from Francis et al. 1993; Kleyn et al. 1993). The proximal phage end clone of YAC 97H12 hybridized to sequences from both chromosome 5p and chromosome 5q (Francis et al. 1993), as did a 5.5-kb plasmid subclone of this end clone, designated "Ox5.5." Ox5.5 also gives a positive signal in Southern blot analyses of chick and hamster genomic DNA, suggesting that it might contain conserved gene sequences (data not shown).

Cosmid libraries were constructed from YACs 79C7 and 17F5. Each library contained ~70 independent clones, which were gridded out in 96-well microtiter dish arrays for ease of further analysis. Forty-two percent of the clones from YAC 79C7 and 68% of those from YAC 17F5 gave a positive signal on screening with Ox5.5. An attempt was made to arrange the cosmids into a contig spanning the region covered by YACs 79C7 and 17F5. The clones were first grouped by sequence-tagged-sites analysis, using the many primer sets that had been previously generated (Francis et al. 1993). For detailed analysis of the clones, a method of fingerprinting with partial *Hin*fI digestion was used. Figure 2 shows the result of such analysis of some of the cosmids from YAC 79C7. The pattern of bands seen with the Ox5.5 positive clones is similar, but not identical, as shown by the arrows. This suggests that the cosmids do arise from distinct genomic regions and do not merely represent a cloning bias in the library construction. Further evidence that the Ox5.5 positive clones are different came from direct sequence analysis of the cosmids, which shows sequence variations between them (data not shown).

One of the positive cosmid clones was subcloned into plasmid, and those subclones that remained positive with Ox5.5 were sequenced and used to screen a human fetal brain cDNA library. These "repeat specific" cosmid sub-

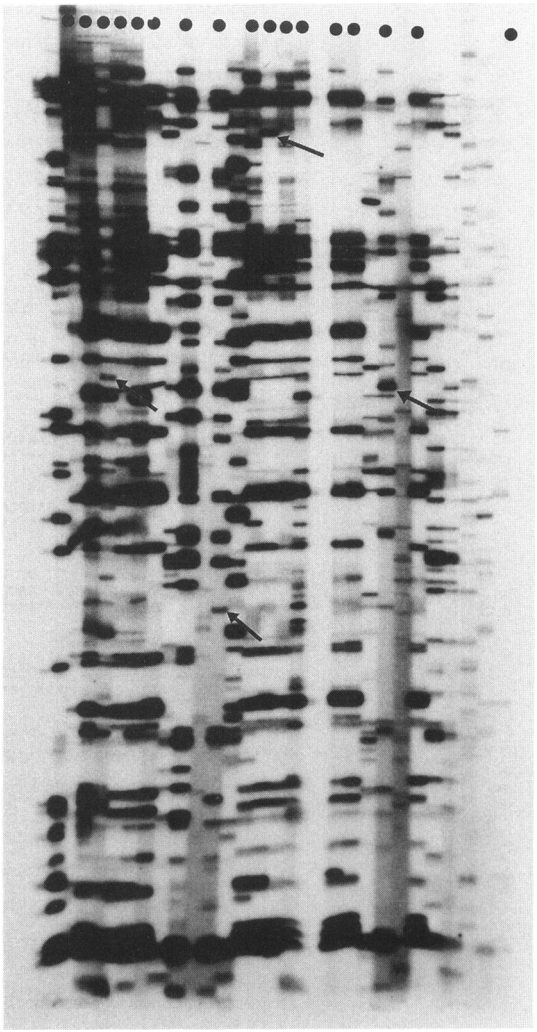
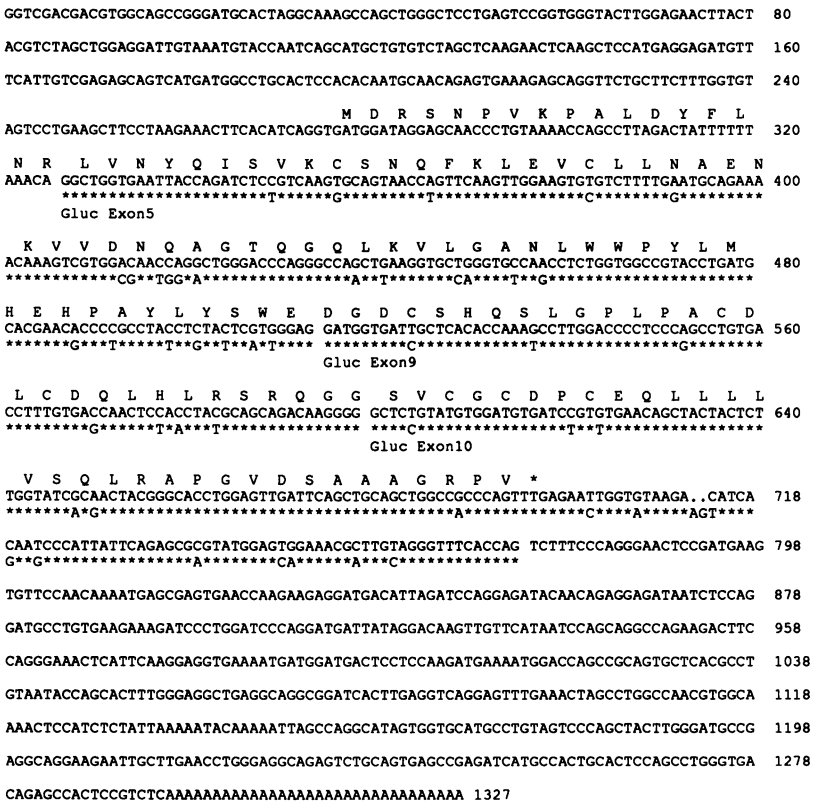
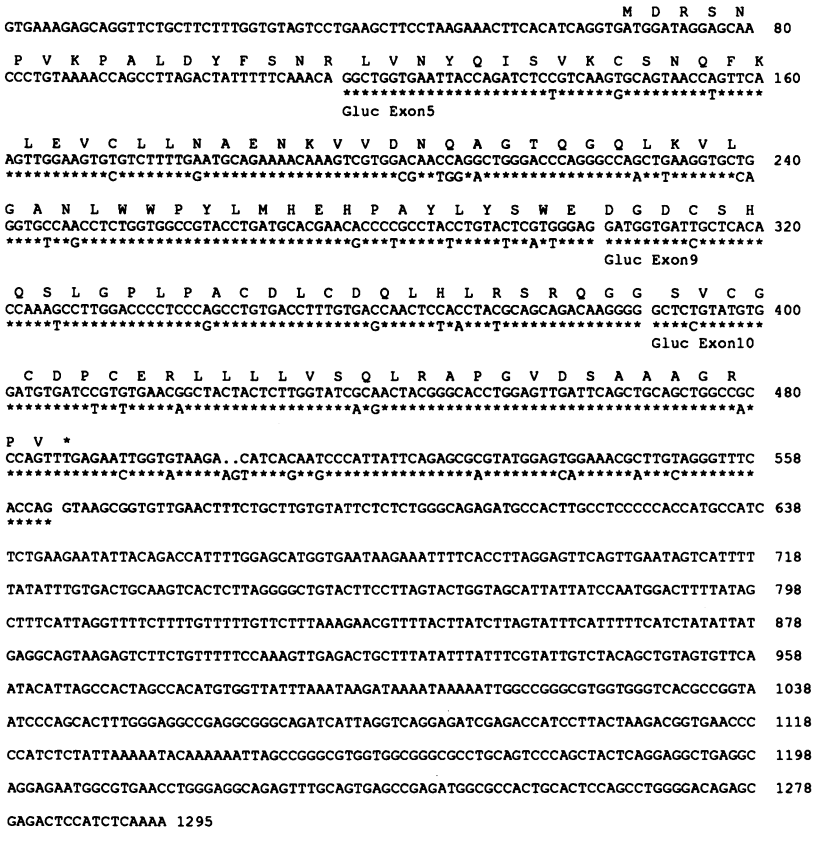


Figure 2 *Hin*fI fingerprinting of some of the human cosmids from YAC 79C7. Cosmids positive on hybridization with Ox5.5 are denoted by a black dot (●). While the pattern of bands seen in these clones is similar, differences are apparent, as illustrated by the arrows.

A


Gluc Exon5
Gluc Exon9
Gluc Exon10

B


Gluc Exon5
Gluc Exon9
Gluc Exon10

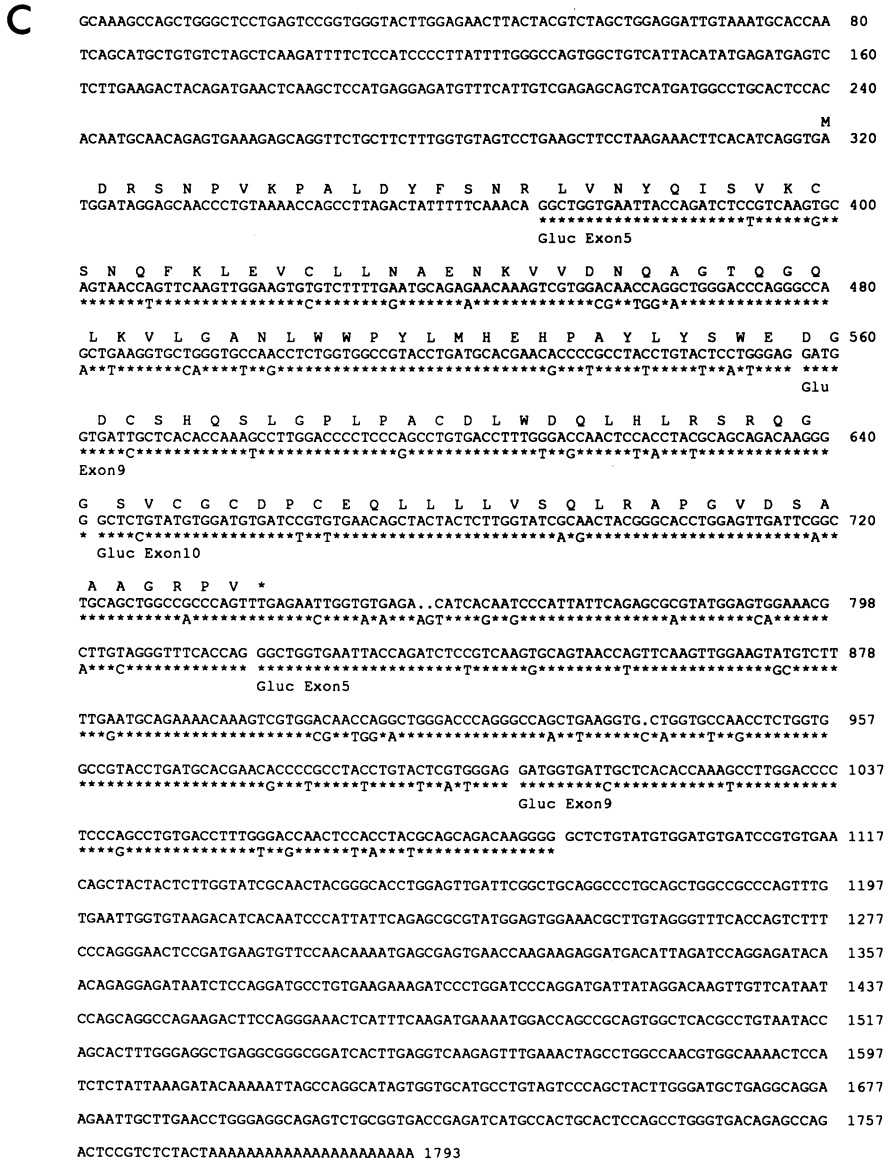


Figure 3 DNA sequences of cDNA clones SMA 3 (*A*), SMA 4 (*B*), and SMA 5 (C). The letters above the cDNA sequences denote the amino acid sequence of their predicted open reading frames. Below the cDNA sequence is shown the alignment of β-glucuronidase exons, with asterisks denoting exact base matches and with letters showing the base differences. Sequence alignments were performed using the PILEUP and PUBLISH programs of the GCG package.

clones gave less complex hybridization signals on genomic screening than did the complete Ox5.5 probe. Fourteen independent cDNA clones were obtained (named "SMA 1–14"). Each of these was hybridized back to pulsed-field gel blots of the YACs from the candidate region (79C7, 11A2, and 17F5). The cDNAs SMA 1–5 hybridized only to YACs 79C7 and 17F5, but not to YAC 11A2. cDNAs SMA 6–14 hybridized to all three YACs. As a key recombination event allows SMA to be mapped proximal to D5S557, a microsatellite marker derived from the proximal end-clone of YAC 11A2 (Francis et al. 1993), cDNAs 6–14 were not analyzed further.

cDNAs SMA 1–5 were completely sequenced. cDNAs SMA 3–5 each showed a complete open reading frame (fig. 3) and contained stretches of sequence that show high homology to the reported β-glucuronidase gene on human chromosome 7 (Oshima et al. 1987). The open reading frame of cDNAs SMA 1 and 2 was incomplete. Again, however, stretches of very high homology to particular exons of the β-glucuronidase gene were evident. A further cDNA clone, denoted "CA1," was obtained on screening an adult brain cDNA library with the Ox5.5 positive plasmid subclones, and it, too, showed high homology to exons of β-glucuronidase. All sequences have been deposited in GenBank.

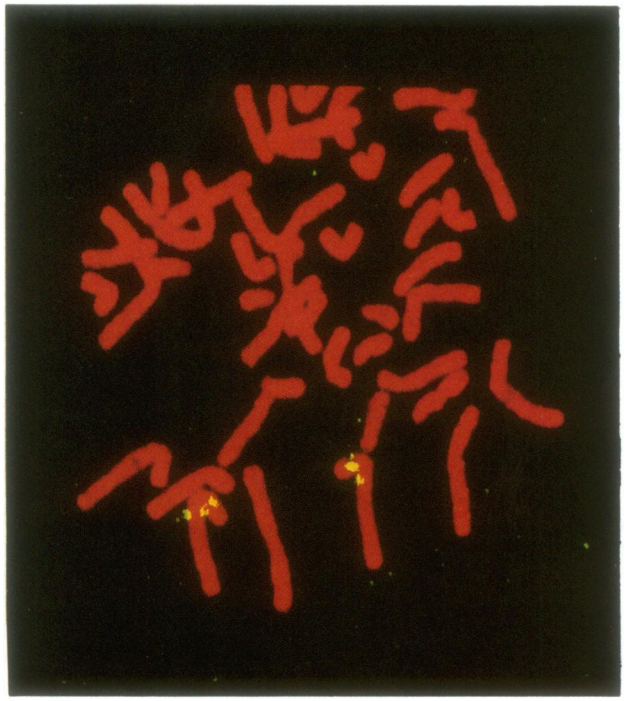


Figure 4 FISH of cDNA CA1 on metaphase chromosomes, showing specific hybridization to chromosome 5p and proximal 5q. An identical FISH pattern was obtained using cDNAs SMA 3–5.

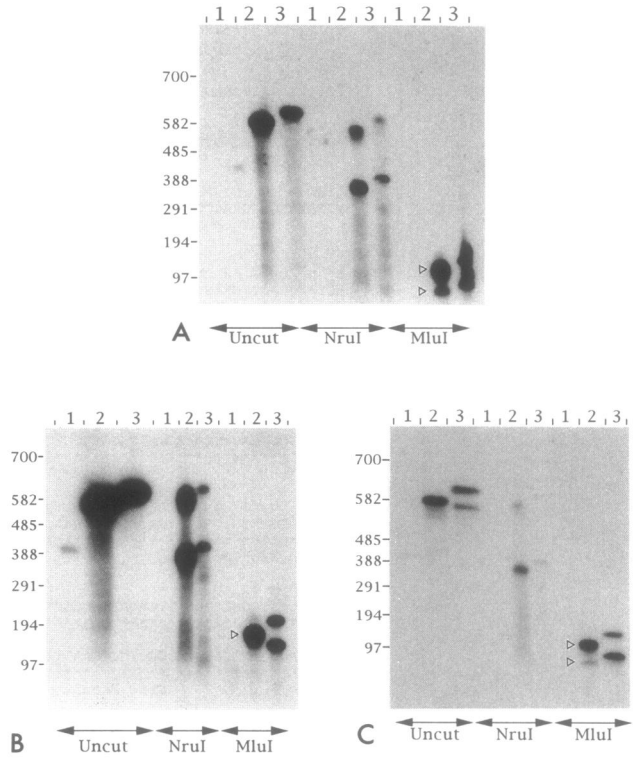


Figure 5 Pulsed-field-gel Southern blots of YACs 11-2A (lanes 1), 79C7 (lanes 2), and 17F5 (lanes 3), undigested or digested with *Nru*I or *Mlu*I and probed with cDNA SMA 3 (*A*), SMA 4 (*B*), and SMA 5 (*C*). Molecular markers (in kb) are shown on the left of each panel. The *Nru*I digests are partial in each case. The differences seen in the *Mlu*I digest patterns with cDNAs SMA 3–5 are not great, but are clearly present and are indicated by arrows.

Localization of cDNAs

FISH analysis.—cDNAs SMA 3–5 and CA1 were each localized by FISH analysis. In each case, the signal obtained was similar to that obtained with the proximal phage end clone from YAC 97H12, with signals clearly evident on chromosome 5p in addition to proximal 5q (fig. 4). No signals were evident on chromosome 7q21.11, where the β-glucuronidase gene is present in single copy.

NruI and MluI Analysis.—To map the cDNAs further within the candidate region, each clone was hybridized to pulsed-field gel blots of *Nru*I- or *Mlu*I-digested YACs spanning the SMA region. As figure 5 shows, each cDNA maps to differently sized *Mlu*I fragments in these overlapping YACs, suggesting strongly that cDNAs SMA 3–5 are distinct transcripts, rather than alternatively spliced fragments of the same RNA transcript.

BamHI Analysis.—Analysis of the Ox5.5 positive cosmids with a series of restriction enzymes showed that few contained sites for the enzyme *Bam*HI. To examine the size and distribution of the repetitive motif in genomic DNA, the cDNA clones were screened against pulsed-field gel blots of *Bam*HI-digested genomic DNA from a number of unrelated individuals. Figure 6 shows the reproducible, highly polymorphic hybridization pattern obtained with CA1. Similar, but not identical, polymorphic hybridization patterns were obtained with cDNAs SMA 3–5. Analysis in one large pedigree showed these polymorphisms to be inherited in a Mendelian fashion (data not shown). The Mendelian inheritance seen in the large-molecular-weight fragments in this family is consistent with the genotype information from several other polymorphic loci that map to chromosome 5q13.3, suggesting that these polymor-

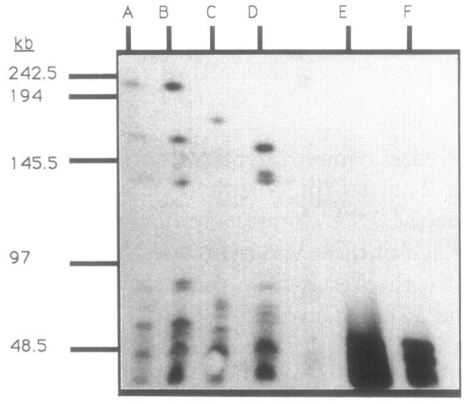


Figure 6 Pulsed-field-gel Southern blots of *Bam*HI-digested genomic DNA from unrelated individuals (lanes A–D) and YACs 79C7 (lane E) and 17F5 (lane F), hybridized with cDNA CA1. Molecular-weight markers (in kb) are shown on the left.

phisms arise from a locus on 5q13, rather than from loci elsewhere. Such a degree of polymorphism may indicate that this region of the genome is susceptible to frequent rearrangements, which may be of relevance to the molecular basis of disease genes mapping within the region (see Discussion). Figure 6 also shows that in contrast to the pattern seen in total human genomic DNA, the fragments identified by Ox5.5 in YACs 79C7 and 17F5 are also large, but <50 kb, perhaps reflecting secondary-structure differences between the genomic and YAC DNAs.

Hybridization against Cosmids from YACs 79C7 and 17F5.—cDNAs SMA 3–5 were also screened against Southern panels of all the cosmids from YACs 79C7 and 17F5 digested with a range of enzymes. Each of the cDNAs gave a positive signal with many of the cosmids. When fragments of the clones that showed no homology to β-glucuronidase were used as probes, similar patterns of hybridization were obtained, again with many positive cosmids identified with each cDNA fragment. Direct sequence analysis of some of the positive cosmids demonstrated that the genomic equivalents of the cDNAs had distinct intron-exon boundary structure and, therefore, do not merely represent processed pseudogenes.

Discussion

In this paper, we have described the characterization of three human fetal brain cDNA clones that map in 5q13 within the SMA candidate region. These clones have been isolated on screening with plasmid clones related to Ox5.5, a probe containing a chromosome 5–specific repetitive sequence, with copies present on 5p and 5q. The three cDNAs are distinct, mapping to different *Mlu*I fragments of YACs isolated in proximal 5q.

Each of the cDNA clones shows homology to a number of the exons of the β-glucuronidase gene, which maps to chromosome 7q21.11. Segments of the β-glucuronidase gene have previously been reported to map to chromosome 5q (Shipley et al. 1993), in addition to chromosomes 6, 7, 20, and 22 and the Y chromosome. The chromosome 5 copies have included exons 6 and 7, the intervening intron, and exon 11. No copies of β-glucuronidase exons 5, 9, and 10 have previously been reported. The active site of the β-glucuronidase enzyme is thought to be encoded in exon 6 (Oshima et al. 1987), which is not represented in any of the clones that map to 5q.

One may speculate on the possible relevance that these β-glucuronidase–like cDNAs in proximal 5q have to the SMA disorder. The phenotype resulting from mutations in β-glucuronidase—i.e., mucopolysaccharidosis type VII, or Sly disease—is clearly very different from that of SMA. The affected individuals are characterized by a range of features, including coarse facies, skeletal deformity, hepatosplenomegaly, and mental retardation. No features of lower-motor-neuron disease are apparent. Mutation analysis of patients with mucopolysaccharidosis type VII has shown that the disease has arisen because of mutations in exons 7 and 12 of the β-glucuronidase gene (Tomatsu et al. 1990, 1991; Shipley et al. 1993). No cases of mucopolysaccharidosis type VII arising because of unequal crossing-over with portions of the many glucuronidase-related sequences elsewhere in the genome have been demonstrated.

Although population studies suggest that SMA is inherited as an autosomal recessive disorder, some features argue against this straightforward mode of inheritance. Several studies report that the segregation ratio seen among siblings, particularly with the milder forms of the disease, is less than the expected .25 (Bundey and Lovelace 1975; Hausmanova-Petrusewicz et al. 1985). Second, the incidence of the disease in second-degree relatives is higher than would be predicted under the autosomal recessive model. Further, different types of SMA are not uncommonly seen to segregate within a family (e.g., see Munsat et al. 1969; Burghes et al. 1994*a*). Becker initially suggested a multiple-allele model to give a possible explanation for some of these phenomena, a model that was extended by Emery (1991), in which a normal dominant allele and at least four rare mutant alleles are proposed. However, studies in four French families with more than one form of SMA segregating (Müller et al. 1992), and our own observations on 11 such families (authors' unpublished observations), reveal that the affected children within a family share common genotypes in the SMA region and are not compound heterozygotes, as would be predicted under the multiple-allele model. A further observation is that the sex ratio observed in SMA deviates markedly from the expected 1:1 for an autosomal recessive disorder, again particularly evident in types II and III of the disease (Hausmanova-Petrusewicz et al. 1984).

In the present study, each of the three ubiquitously expressed cDNA clones isolated shows a highly polymorphic genomic arrangement between individuals. It is possible that both the repetitive nature of these gene sequences and their clustering within a relatively small (<1 Mb) interval provides a potential mechanism for aberrant recombination events, either by unequal sister-chromatid exchange or by gene conversion, similar to the situation suggested to underlie rearrangements in the 21-hydroxylase gene cluster on chromosome 6p (Sinnott et al. 1990). These recombination events may silence or disrupt nearby genes and may be of relevance to the pathogenesis of SMA. If such complex rearrangements are indeed involved in SMA, this may give an explanation of the observed features of the inheritance in the disorder. It is of interest that copies of β-glucuronidase exons 3 and 11 have been mapped to the Y chromosome, which may provide an explanation of the male bias. A further observation is that expression of the mouse β-glucuronidase gene is exquisitely responsive to androgen, the androgen-responsive elements mapping within intron 9 of the gene (Lund et al. 1991). While β-glucuronidase exon 9 motifs are present in the cDNAs iso-

lated in the present study, it is not yet known if the androgen-responsive intronic sequences map in any of the chromosome 5q13 genes isolated. It is of further interest that each of the cDNA clones described in this paper maps in the region in which deletions and loss of heterozygosity have recently been detected with polymorphic microsatellite markers in a proportion of French SMA individuals (Melki et al. 1994).

Acknowledgments

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