chronic defect in remnant clearance) also have substantially lower HDL cholesterol concentrations than normal animals, suggesting a functional connection between HDL and remnant metabolism.

In conclusion, the results presented in this study provide additional evidence for a physiological dual role of LRP in the metabolism of lipoproteins and proteases. Furthermore, our results have demonstrated the usefulness of adenovirus-mediated gene transfer to overexpress a dominant negative regulator and to study the physiological consequences of the transient inactivation of the target protein in an intact animal. Such an approach may be generally applicable for the in vivo inactivation and study of other developmentally essential genes (such as growth factors, signaling receptors, or transcription factors) for which dominant negative mutants or antagonists exist.

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18. Recombinant adenovirus expressing the full-length rat RAP cDNA (AdCMV-RAP) was constructed by a modification of the procedure described in (*17*). Briefly, a plasmid containing the left inverted repeat and sequences from map unit 0 to 1.3 of adenovirus 5 (Ad5), a cytomegalovirus (CMV) promoter, the RAP cDNA, and the 3′-untranslated region of bovine growth hormone (bpA) was linearized at a unique Xba I site downstream of bpA and ligated to adenovirus sequences extending from map units 9.1 to 100 of the Ad5 derivative, AdRR5. 293 cells were transfected with the ligation mixture, and recombinant viruses containing RAP were identified by polymerase chain reaction. AdCMV–β-Gal, AdCMV-Luc, and procedures of virus preparation were as described (*17*). Three independent AdCMV-RAP isolates were used and gave equivalent results. Virus preparations were titrated on 293 cells. Plaque-forming units (PFU) were related to viral particle numbers and were similar for independent virus preparations (2×10^{11} particles $\approx 5 \times 10^9$ PFU).
19. Human methylamine-activated α_2M and bovine asialofetuin (Sigma) were iodinated by the Iodogen procedure (*13*). Wild-type or LDLR$^{-/-}$ C57BL/6J × 129SvJ hybrid male mice were bred in house and fed ad libitum throughout the course of the experiment (Teklad 4% Mouse/Rat Diet 7001, Harlan Teklad Premier Laboratory Diets). Before virus injection and turnover studies, animals were anesthetized by intraperitoneal sodium pentobarbital (Nembutal) injection (~80 μg per gram of body weight). Viruses or iodinated protein were slowly injected into the external jugular vein in a total volume of 200 to 250 μl of tris-buffered saline. Animal care and experimental procedures involving animals were conducted in accordance with institutional guidelines.
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De Novo and Inherited Deletions of the 5q13 Region in Spinal Muscular Atrophies

Judith Melki, Suzie Lefebvre, Lydie Burglen, Philippe Burlet, Olivier Clermont, Philippe Millasseau, Sophie Reboullet, Bernard Bénichou, Massimo Zeviani, Denis Le Paslier, Daniel Cohen, Jean Weissenbach, Arnold Munnich*

Spinal muscular atrophies (SMAs) represent the second most common fatal autosomal recessive disorder after cystic fibrosis. Childhood spinal muscular atrophies are divided into severe (type I) and mild forms (types II and III). By a combination of genetic and physical mapping, a yeast artificial chromosome contig of the 5q13 region spanning the disease locus was constructed that showed the presence of low copy repeats in this region. Allele segregation was analyzed at the closest genetic loci detected by markers C212 and C272 in 201 SMA families. Inherited and de novo deletions were observed in nine unrelated SMA patients. Moreover, deletions were strongly suggested in at least 18 percent of SMA type I patients by the observation of marked heterozygosity deficiency for the loci studied. These results indicate that deletion events are statistically associated with the severe form of spinal muscular atrophy.

Proximal spinal muscular atrophies (incidence: 1 out of 6000 newborns) (*1–4*) are characterized by degeneration of anterior horn cells of the spinal cord, leading to progressive symmetrical limb and trunk paralysis associated with muscular atrophy. The childhood spinal muscular atrophies are divided into types I (Werdnig-Hoffmann disease), II, and III (Kugelberg-Welander disease) on the basis of age of onset, milestones of development, and life span (*5*). By means of linkage analysis, we and others have shown that all three forms of spinal muscular atrophy map to chromosome 5q11.2–q13.3 (*6–9*).

J. Melki, S. Lefebvre, L. Burglen, P. Burlet, O. Clermont, S. Reboullet, B. Bénichou, M. Zeviani, A. Munnich, Unité de Recherches sur les Handicaps Génétiques de l'Enfant, INSERM U-393, Hôpital des Enfants-Malades, 75743 Paris, France.
P. Millasseau and J. Weissenbach, Genethon, Evry, France.
D. Le Paslier and D. Cohen, Centre d'Etudes du Polymorphisme Humain (CEPH), Paris, France.

*To whom correspondence should be addressed.

We have recently identified two DNA markers flanking the SMA gene at loci *D5S629* and *D5S637* (*10*). This interval [2 centimorgans (cM)] was cloned into a 4-Mb yeast artificial chromosome (YAC) contig spanning the disease locus by YAC chromosomal walking [Fig. 1A and (*11*)]. Among the 28 DNA markers derived from the YAC contig, 9 also mapped to chromosome 5p, suggesting partial duplication of the 5q13 region on chromosome 5p. Moreover, nine markers mapped to more than one locus within the 5q13 region, indicating the presence of low copy repeats on chromosome 5q13 (*12*). Indeed, taking advantage of DNA polymorphisms, we observed that four of them revealed two loci [C212 (*D5F149S1*, *-S2*), C272 (*D5F150S1*, *-S2*), C271 (*D5F148S1*, -S2), and C171 (*D5F151S1*, *-S2*)] and one of them three loci [C161 (*D5F153S1*, *-S2*, *-S3*)] on the monochromosomal hybrid HHW105 (*13*).

Haplotype analysis and homozygozity mapping of 10 SMA families, to identify recombinant events between the disease gene and the flanking loci *D5S629* or *D5S637* (*10*), was done with polymorphic DNA markers derived from the YAC contig (*14*). No recombination event was detected between the most polymorphic loci identified by markers C212 and C272 and the SMA locus, indicating that these markers are close to the disease gene.

To test the hypothesis that the low copy repeats of the 5q13 region might trigger large-scale chromosomal rearrangements, we analyzed the allele segregation at loci recognized by markers C212 and C272 in 201 nonconsanguineous SMA families (*15*). The absence of one parental allele at these loci was observed in eight affected children from seven unrelated SMA families. Four children had type I, two had type II, and two had type III spinal muscular atrophy (Fig. 2). A de novo rearrangement involving one of the two loci detected by C272 and C212 was observed in the chromosome of one SMA type I patient (Fig. 2, family 7). We obtained consistent results by using different pairs of oligonucleotide primers for both markers. False paternity or missampling was ruled out by complete haplotype analysis of the families. No abnormal allelic segregation was observed with polymorphic flanking markers (*16*). These results suggest that a rearrangement occurred on one mutant chromosome which encompassed the disease gene in seven unrelated patients.

Because the number of patients harboring rearrangements may have been underestimated by lack of informativity, the number of distinct C212- and C272-specific polymerase chain reaction (PCR) fragments was analyzed in our SMA series and in controls [Fig. 3 and (*17*)]. A total of 16 out of 90 (18%) type I patients had one single C272 amplification product, compared with 1 out of 81 (1%), 1 out of 30 (3%), and 0 out of 59 (0%) in type II, type III, and controls, respectively ($P < 0.001$). Accordingly, the proportion of SMA type I patients bearing two C272 amplification products (55/90; 61%) also significantly differed from that of parents (69/180; 38%) and controls (12/59; 20%, $P < 0.001$). Similar results were obtained with marker C212 (*17*). Subsequently, 20 SMA type I families whose probands had a single amplification product, as determined with markers C212 or C272, were further analyzed with marker C161. In 2 out of 20 families, a de novo

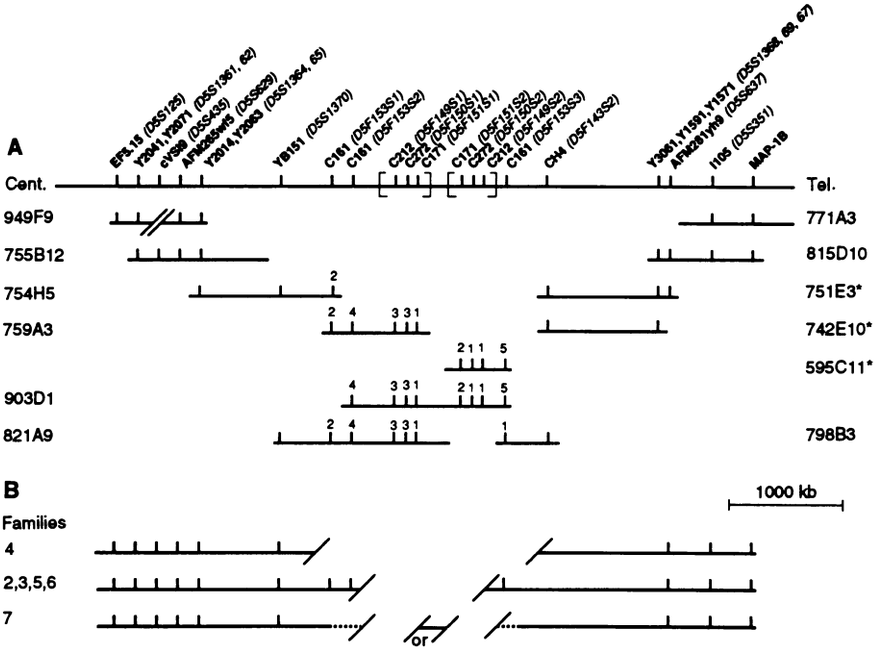

Fig. 1. YAC contig spanning the SMA region and genetic map of deletions in SMA patients. (**A**) Organization of the YAC contig. DNA markers and YACs are indicated above and below the genomic map, respectively. Brackets covering the clusters C212-C272-C171 indicate that the order of markers within the centromeric and the telomeric clusters is unknown. D-segments are in parenthesis; asterisks indicate chimeric YAC clones. Cent., centromere; Tel., telomere. The numbers above the YAC map indicate the genotypes at polymorphic loci C161, C212, C272, and C171. To confirm the extension of the YAC contig over the entire region, we used the two loci detected by each of the polymorphic microsatellite markers C212, C272, and C171 and the three loci detected by polymorphic marker C161 to compare the haplotype of the YAC clones with that of the human donor. YAC 759A3 was not allelic to 595C11, and YAC 798B3 was allelic to 903D1 at the telomeric locus detected by marker C161. The position of these markers in the YAC contig was in agreement with the genetic cartography of the chromosomal deletions. (**B**) Genetic map of deletions in SMA patients. In family 7, the deletion involves either the centromeric or the telomeric block C212-C272-C171.

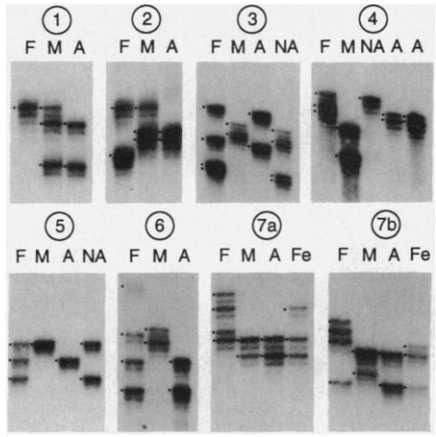

Fig. 2. Parental allele contribution in SMA patients as determined by microsatellite DNA markers C212 and C272. The figure shows family studies based on marker C272 (families 1, 2, 5, and 7b) and C212 (families 3, 4, 6, and 7a). The SMA patients belonged to type I (families 3, 5, 6, and 7), type II (families 1 and 2), or type III (family 4). The parental noncontribution is of paternal origin in families 1 and 2 and of maternal origin in families 3, 4, 5, and 6. In families 4 and 5, the nonaffected siblings (NA) are heterozygous for the disease locus as determined by flanking markers (*16*). In family 7, note the incomplete contribution of the father to his affected child with C212 (7a) and C272 (7b) compared with the haploidentical fetus (Fe) with flanking markers (*16*). F, father; M, mother; A, affected; NA, nonaffected; Fe, fetus. Dots indicate allelic fragments.

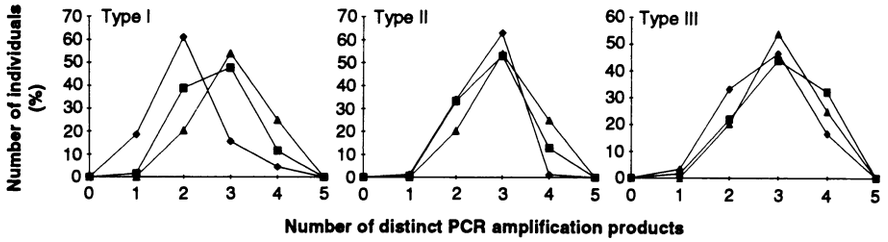

Fig. 3. Number of amplification products revealed by marker C272 in SMA patients, parents, and controls. A total of 90 type I, 81 type II, and 30 type III SMA probands, their parents, and 59 controls were investigated. We ruled out the possibility that heterozygosity deficiency was due to distant consanguinity, because the estimation of shared fragments in the parents of SMA patients was not statistically different from that observed in 20 unrelated control families. ◆, affected; ■, parents; ▲, controls.

rearrangement removing two out of three loci detected by marker C161 was observed (Fig. 4, families 8 and 9). These results support the idea that heterozygosity deficiency in type I spinal muscular atrophy is due to loss of alleles at these loci.

To characterize the extent of the 5q13 region involved in the rearrangements, we tested all polymorphic DNA markers derived from the YAC contig (*14*) in SMA families showing evidence for abnormal parental contribution (Fig. 1B). The smallest rearrangement occurred within the region bordered by loci detected by C161 and C212-C272-C171, suggesting that the SMA locus lies within a 1.2-Mb region (Fig. 1). However, because of sequence identity of the regions flanking the C212-C272 CA-repeats, we were unable to determine which of the two loci was lacking in the proband harboring a single locus rearrangement (Figs. 1B and 2, family 7).

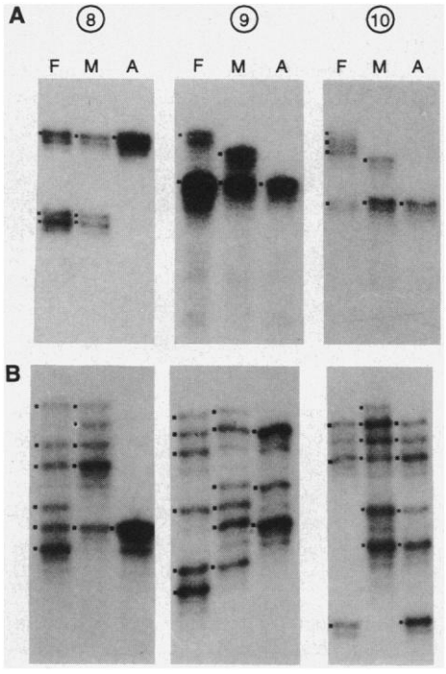

Fig. 4. Evidence for de novo deletions seen with microsatellite DNA marker C161 in SMA type I patients. (**A**) Segregation of alleles detected by marker C272 shows no evidence either for or against a deletion in families 8, 9, and 10. Note that the patients have a single amplification product. (**B**) Segregation of alleles revealed by marker C161 detects de novo deletions in families 8 and 9, as the proband inherited only one locus from both parents (family 8) or from the father (family 9). In family 10, the detection of six PCR amplification products in the proband excludes a deletion encompassing the three loci detected by C161. F, father; M, mother; A, affected. Dots indicate allelic fragments.

A λ phage library of YAC 595C11 was constructed and clone L-132, containing the C272 marker, was used as a probe for Southern (DNA) blot analysis of SMA families showing abnormal parental contribution (*18*). Gene dosage and restriction fragment length polymorphism (RFLP) analysis indicate the presence of either inherited or de novo deletions (Fig. 5).

Our study provides direct genetic and physical evidence for large-scale deletions encompassing the disease locus in nine unrelated SMA patients. Moreover, the presence of deletions of the 5q13 region is supported by heterozygosity deficiency statistically associated with the severe form of spinal muscular atrophy (type I). Deletions were occasionally observed in type II or type III SMA patients, suggesting that distinct allelic mutations might account for the variable clinical expression of the disease and confirming that the gene or genes for all three forms map to the same region. Duchenne and Becker muscular dystrophies can also result from gene deletions, but in these cases a mechanistic difference in the types of the deletions has been found (*19*), whereas no such data is available for spinal muscular atrophy.

This study also demonstrates that deletions can occur de novo, a feature that might account for the low segregation ratio in spinal muscular atrophy (*20, 21*). De novo deletions of the 5q13 region might

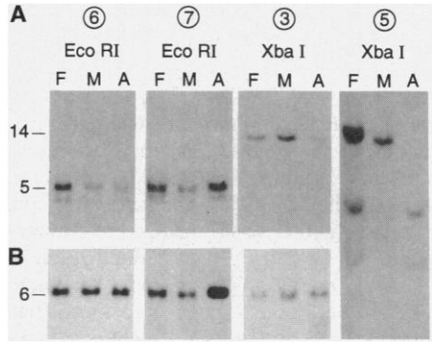

Fig. 5. Gene dosage and RFLP analysis of the 5q13 region with the L-132 phage clone in SMA type I patients. Total human DNA from SMA families showing a lack of allele contribution from one parent were digested with either Eco RI (families 6 and 7) or Xba I (families 3 and 5). Filters were consecutively hybridized with L-132 (**A**) and JK53 probes (**B**). Gene dosages were determined by densitometric scanning of the hybridization signals in families 3, 6, and 7. In family 6, both the mother and the affected child had a 50% reduction in band intensity, which indicates that the deletion was inherited. A significant decrease in L-132 band intensity was observed in the probands of families 7 and 3 compared with their parents, which indicates that the deletions were de novo. In family 5, Xba I RFLP analysis showed that the proband had not inherited the maternal allele.

also account for the apparent genetic heterogeneity of the disease (*8, 22*) when normal and affected siblings share common haplotypes using flanking markers. This observation is also relevant to genetic counseling. Reliance on haploidentity of a fetus and an affected sibling determined by flanking markers might lead to prenatal misdiagnoses when a de novo deletion is involved.

The YAC contig reported here contains all known repeated sequences of the 5q13 region and shows that the size of the region encompassing the disease locus is substantially larger than previously reported (*23, 24*). The presence of low copy repeats on chromosome 5q13 may account for the instability of this region and trigger frequent deletions by means of unequal crossing-over events in spinal muscular atrophy. Finally, the characterization of the smallest deletions in spinal muscular atrophy will contribute to identification of the disease gene or genes.

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11. YAC libraries from CEPH were screened by PCR with microsatellite DNA markers according to the three-dimensional (3D) PCR procedure [E. D. Green and M. V. Olson, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1213 (1990)]. YAC genotypes were established by electrophoresis of PCR products on denaturing polyacrylamide gels [J. Hazan *et al.*, *Genomics* **12**, 183 (1992)]. YAC size was estimated by pulsed-field gel electrophoresis (PFGE) with a Pharmacia-LKB apparatus. YAC chromosomal walking was performed with the ALU PCR method [I. M. Chumakov *et al.*, *Nature Genet.* **1**, 222 (1992)]. Sequence tagged sites (STSs) and microsatellite DNA markers were isolated from the selected YACs after purification by PFGE through a 1% Seaplaque GTG agarose gel. After B-agarase digestion, a total of 300 ng of YAC DNA was digested with Sau 3A, partially filled, and subcloned into the partially filled Sal I site of M13 bacteriophage. M13 clones containing CA- or CT-repeats were detected by plaque hybridization with radiolabeled $(CA)_{20}$ and $(CT)_{20}$ oligonucleotides. DNA templates from (C-A)– or (C-T)–positive clones were then sequenced as described [L. M. Smith, J. Z. Sanders, R. J. Kaiser, *Nature* **321**, 674 (1986)]. Markers were assigned to their chromosomal location by Southern blot analysis and PCR amplification with primers flanking tandem repeats of DNAs from the panel of somatic cell hybrids of chromosome 5 (*13*). Polymorphic loci were detected by testing five unrelated controls. The nonpolymorphic markers were used as STSs.
12. Among the 28 identified DNA markers, 9 were excluded from the chromosomal walking because they were also present on chromosome 5p. The remaining 19 markers were used to select overlapping YACs. Location of STSs and microsatellites derived from YACs spanning the SMA region (an asterisk indicates polymorphic markers): (i) multiple loci on chromosome 5 (5p + 5q): 755C1 (*D5F138S1*), Y2061 (D5F139S1, -S2), Y2091 (D5F140S1, -S2), AFM157xd10 (D5S1472),* Y3021 (D5F141S1), Y3011 (D5F142S1), CH4 (D5F143S1, -S2), Y554 (D5F144S1), C132 (D5F145S1)*; (ii) multiple loci within the 5q13 region: 755C1 (D5F138S2, -S3), Y3053 (D5F146S1, -S2), YD1 (D5F147S1, -S2), Y3021 (D5F141S2, -S3), Y3011 (D5F142S2, -S3), AFM157xd10 (D5S1472),* Y554 (D5F144S2, -S3), C271 (D5F148S1, -S2),* C212 (D5F149S1, -S2),* C272 (D5F150S1, -S2),* C171 (D5F151S1, -S2),* YC10 (D5F152S1, -S2), C161 (D5F153S1, -S2, -S3),* C132 (D5F145S2, -S3),* SA1 (D5F154S1, -S2); and (iii) probable single human sequences: Y2041 (D5S1361),* Y2071 (D5S1362),* Y2011 (D5S1363),* Y2014 (D5S1364),* Y2063 (D5S1365),* Y2062 (D5S1366), Y1571 (D5S1367), Y3061 (D5S1368), Y1591 (D5S1369), YB151 (D5S1370). The sequences of PCR amplification primers are deposited in Genome Data Base and are for C212: 5′-CCTCCACCCTGGGTGATAAG and 5′-GCTGATGAAGTTGTAGGAGGC; for C272: 5′-TAGAGACGGGGTTTCGGCAT and 5′-GATCTGCCTTCCTTCCTGC; and for C161: 5′-GGCTTCCTCCTGAGTATGCA and 5′-GTTTCACTGGATGGAACGGC.
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14. The polymorphic microsatellite DNA markers generated from the YAC contig and mapped only to the 5q13 region are C212, C272, C171, C161, C271, Y2041, Y2071, Y2011, Y2014, Y2063, and YB151. The last six markers only recognized a single locus. Amplification conditions were as follows: denaturation at 94°C, annealing at 55°C, and extension at 72°C, 1 min each for 30 cycles. The map position of these markers is shown in Fig. 1A.
15. In this study 201 nonconsanguineous SMA families were included. On the basis of diagnostic criteria of the International SMA Consortium (*5*), families were selected and placed into one of the three subgroups (type I, II, or III). Ninety infants belonged to type I, 81 to type II, and 30 to type III. Two large CEPH reference families, 22 healthy families composed of two parents and one offspring, and 60 unrelated healthy individuals were used as controls. To prevent bias in the statistical analysis of our results, we included only one affected child per family in this study. No linkage disequilibrium was observed.
16. J. Melki *et al.*, data not shown.
17. The number of distinct PCR amplification products observed with C272 and C212 in SMA type I, II, and III patients, their parents, and controls are as follows ([*n*] indicates the number of individuals tested). (i) Marker C272; patients: type I [90]—1 (18.5%), 2 (61%), 3 (15.5%), 4 (4.5%); type II [81]—1 (1%), 2 (34%), 3 (63%), 4 (1%); type III [30]—1 (3%), 2 (33%), 3 (46.5%), 4 (16.5%). Parents: type I [180]—1 (1.5%), 2 (38.5%), 3 (47.5%), 4 (11.5%); type II [162]—1 (0.5%), 2 (33%), 3 (53%), 4 (13%); type III [59]—1 (1.5%), 2 (22%), 3 (44%), 4 (32%). Controls [59]—1 (0%), 2 (20%), 3 (54%), 4 (25%). (ii) Marker C212; patients: type I [90]—1 (13%), 2 (66.5%), 3 (13%), 4 (6.5%); type II [76]—1 (2.5%), 2 (35.5%), 3 (59%), 4 (2.5%); type III [30]—1 (3%), 2 (23%), 3 (66.5%), 4 (6.5%). Parents: type I [180]—1 (1.5%), 2 (42%), 3 (45.5%), 4 (10.5%); type II [150]—1 (0.5%), 2 (30%), 3 (53%), 4 (16%); type III [59]—1 (0%), 2 (23.5%), 3 (52.5%), 4 (23.5%). Controls [60]—1 (0%), 2 (18%), 3 (60%), 4 (21.5%).
18. Total yeast DNA from YAC clone 595C11 was purified and partially digested with Sau 3A. DNA in the size range of 12 to 23 kb was excised after 0.5% Seaplaque GTG agarose gel electrophoresis and precipitated with ethanol after B-agarase digestion. After partial fill-in of the Sau 3A site, DNA was subcloned at the partially filled Xho I site of bacteriophage FIX II (Stratagene). Clones of λ containing the markers C212 (L-51), C272 (L-51, L-132), and C171 (L-5, L-13) were tested for RFLPs. Five unrelated control DNAs were digested with restriction enzymes Eco RI, Pst I, Msp I, Bgl II, Xba I, Hind III, or Rsa I, separated by electrophoresis on an 0.8% agarose gel for Southern blotting, and hybridized with radioactively labeled probes. The JK53 DNA fragment (*D5S112*) was used as an internal control probe for gene dosage analysis. Autoradiographs were scanned at 600 nm in a computerized densitometer (Hoefer Scientific Instruments, San Francisco).
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