frames, as used for these dynamic studies. $[Ca^{2+}]_i$ was calculated for these studies by summing all pixels in the cytoplasm in the 340- and 380-nm images. This process would be expected to decrease the uncertainty by 67 times for a typical cell.

31. S. B. Abramson, K. E. Fogarty, F. S. Fay, in preparation. Cell lengths were determined by defining the central axis of the cell in each image of a series with interactive computer graphics software. The number of pixels that constituted this axis was then converted to an actual measurement of cell length by reference to a conversion scale (pixels per micrometer) with correction for the aspect ratio of each pixel within the axis. Fluorescence intensities (at each excitation wavelength) were calculated with a joystick-driven, interactive graphics software routine to define areas of interest within each image (for example, cell boundaries and the nuclear-cytosol interface). All pixel intensities within the cytosol and nucleus were summed. The fluorescence ratios were then calculated for the nucleus or cytoplasm as a whole by dividing the total fluorescence (measured in a particular area at 340 nm) by the same measure made at 380 nm. Ratios based on division on a pixel-by-pixel basis were not calculated during contraction as such images are subject to severe distortion due to the motion of the cell between consecutive 340- and 380-nm images. As fluorescence could not be measured simultaneously at each wavelength, a ratio was determined by the division of the intensities within a given 340-nm image by those resulting from an interpolation between the 380-nm images that preceded and followed this image.

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A New Probe for the Diagnosis of Myotonic Muscular Dystrophy

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Myotonic muscular dystrophy (DM) is the most common muscular dystrophy, affecting adults as well as children. It is inherited as an autosomal dominant trait and is characterized by variable expressivity and late age-of-onset. Linkage studies have established the locus on chromosome 19. In order to identify tightly linked probes for diagnosis as well as to define in detail the DM gene region, chromosome 19 libraries were constructed and screened for restriction fragment length polymorphisms tightly linked to DM. A genomic clone, LDR152 (D19S19), was isolated that is tightly linked to DM; recombination fraction = 0.0 (95% confidence limits 0.0–0.03); lod score, 15.4.

MYOTONIC DYSTROPHY (DM) IS the most common form of adult and childhood muscular dystrophy. It is inherited as an autosomal dominant trait located on chromosome 19. There have been no documented cases of new mutation (*1*). Multiple very large families have been evaluated clinically and genotyped by means of both serum and DNA polymorphisms in a linkage strategy designed to identify loci tightly linked to the disease. Six large clinically studied DM families were used in the linkage analysis. Thus far over 400 individuals have been genotyped. The apolipoprotein C2 (ApoC2) locus has been closely linked to DM (*2, 3*). Although approximately 96 to 98% accurate for diagnosis, it is too far from the DM locus to be a starting point for the physical techniques of chromosome walking and/or hopping to isolate the gene. We therefore screened restriction fragment length polymorphisms (RFLPs) on chromosome 19 (CH19) in these families in order to identify a more tightly linked marker. New probe testing was dependent upon the absence of detectable crossover events and was focused toward defining the DM gene region rather than mapping CH19.

A flow-sorted, genomic DNA library enriched for CH19, was prepared from a human lymphoblast cell line established by Epstein-Barr virus transformation of white blood cells from a normal female. A bivariate flow karyogram of a typical preparation is shown in Fig. 1A. Since CH19 represents only 2.2% of the human genome and only 1.1% of the preparation in Fig. 1A, chromosome preparations were enriched by fractionation on linear rate zonal sucrose gradients. For comparison, analyses of fractions obtained from both the dense and less dense regions of a typical gradient demonstrate enrichment of chromosomes of groups B and C in one and enrichment of chromosomes in groups E, F, and G in the other (Fig. 1, B and C, respectively). The inherent sorting efficiency of the fractions from Fig. 1C would therefore be much greater for CH19 than the total complement (Fig. 1A). In addition to the size enrichment, the sucrose gradients removed the interphase nuclei from the chromosome fractions since nuclei pellet through the gradient during the first few minutes of centrifugation.

Fractions enriched in group F were pooled from six gradients and submitted to preparative bivariate flow sorting. Fluorescence analysis (630× power) of a sample of sorted chromosomes indicated that 53/57 (93%) were CH19, 3/57 were CH21, and 1/57 was an unidentifiable fragment. Therefore, cloning the DNA derived from sorting CH19 under these same configurations should significantly enrich for clones localized to CH19.

Genomic DNA was prepared from 1.3×10^6 flow-sorted CH19 and digested completely with Hind III. This DNA was cloned into the Hind III insertion site in Charon 21A. A total of 5.0×10^6 plaque-forming units were obtained from the packaging reaction. After screening phage plaques that had been transferred to filters (*4*) with total human DNA, small preparations (mini-lysates) from each of 403 nonhybridizing clones were digested with Hind III (Fig. 2A). Of 69 inserts determined to be free of human repeat DNA and ranging in size from 930 to 7800 bp, 30 were screened against hybrid cell panels (Fig. 3). Four regions of CH19 were distinguished and these represented approximately four equal increments of the chromosome.

Clone LDR152 (Fig. 2A, lane 5) is a 1.60-kbp Hind III fragment that maps to the proximal long-arm of CH19 (Figs. 2B and 3). The insert was not digested by Bam HI, Bst NI, Bst XI, Eag I, Eco RI, Eco RV, Hind III, Not I, Pst I, Sac I, Sac II, Sal I, Sfi I, Spe I, Taq I, Xba I, Xho I, or Xmn I. Screening of LDR152 for DNA polymorphisms was successful for Msp I and Pst I (Fig. 4A). In the case of the Msp I polymorphism, the alleles were 2.3 and 1.3 kb in size (gene frequencies were 0.15 and 0.85, respectively). The alleles for the Pst I polymorphism were 19 and 11 kb in size (gene frequencies were 0.14 and 0.86, respectively). Gene frequencies were calculated on the

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basis of a Caucasian population of 76 individuals. These polymorphisms have been assigned the number D19S19 (*5*). LDR152 is not polymorphic for Bam HI, Bgl I, Bst NI, Bst XI, Eco RI, Eco RV, Hind III, Sac I, Stu I, Taq I, or Xmn I (*5*).

Segregation of D19S19 in informative DM families has yet to demonstrate a crossover with DM for either polymorphism. Haplotypes were constructed, and the data were analyzed with the computer program LIPED (*6*). At-risk individuals were assigned probabilities of inheriting the DM gene on the basis of their age at time of examination. The age-of-onset curve used was as described by Harper (*7*).

The maximum recombination frequency ($\hat{\theta}$) is 0.0 (95% confidence limits, 0.0–0.03) with a lod score of 15.4. An illustration of the segregation of the Msp I alleles in a large multigenerational DM pedigree is in Fig.

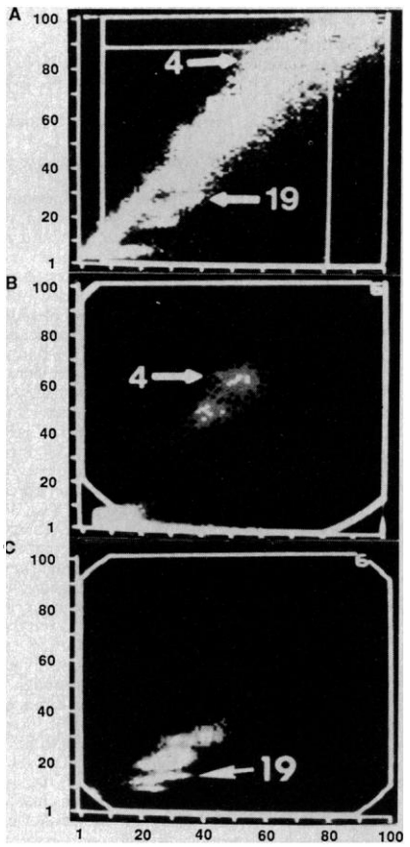

Fig. 1. Bivariate flow histogram displays of chromosomes from a lymphoblast cell line VL324. The total flow karyotype (**A**) and flow karyotypes of sucrose gradient fractions from the same sample with an enriched B- and C-group fraction (**B**) and an enriched fraction of chromosomes from groups E, F, and G (**C**) are shown. Cell growth and preparation of chromosome fractions were as described (*15–17*). Chromosomes were sorted with a modified Ortho 50-H Cytofluorograph. The chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI) and chromomycin A_3 and loaded into a 30-ml syringe that was kept cool by wrapping in Tygon tubing through which ice-cold ethanol was recirculated. With highly enriched fractions, the actual flow and sorting rates were up to 1200 per second and 200 per second, respectively, as generated by a motor-drive syringe pump. Chromosomes were illuminated sequentially with the 351/364-nm line of a Coherant model 90-5 argon ion laser operating at 200 mW, and the 457.9-nm line of a Lexel model 95 argon ion laser operating at 100 mW. Right angle fluorescence signals were collected and data compiled with software in the Ortho 2140 Data Handling System. Photographs were taken directly off the cathode-ray tube display with a Polaroid camera. Sheath buffer was the same as hypotonic chromosome buffer (*15*) with the exception of the removal of the ribonuclease. Chromosomes were collected in polypropylene tubes on dry ice.

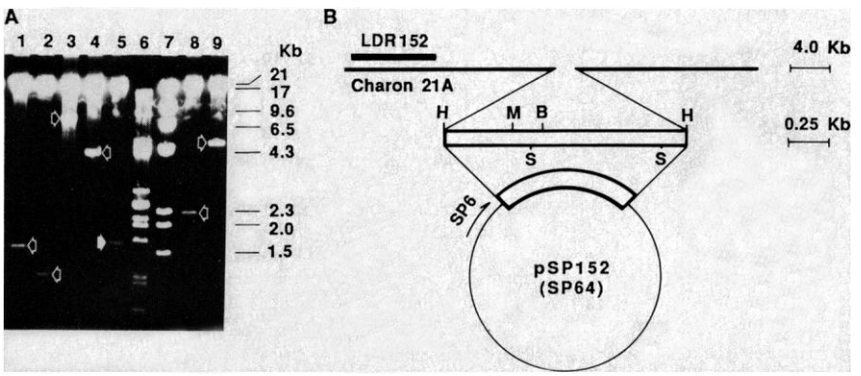

Fig. 2. Mini-lysate preparations of putative CH19-containing Charon 21A clones digested with Hind III and separated by electrophoresis on 1% agarose gels (**A**). Cloned inserts are indicated by arrowheads. (Lanes 6 and 7) Pst I and Hind III/Kpn I–digested λ DNA standards. (Lane 5) LDR152 (solid arrowheads). (**B**) A limited restriction digestion map of LDR152 (M, Msp I; B, Bgl I; S, Sau 3A). Flow-sorted chromosomes were recovered from a 24-hour sort at a flow rate of 1100 events per second and an actual sort rate of 200 per second. Chromosomes were pelleted in an SW50.1 rotor for 3 hours at 45,000*g* and the supernatant removed. DNA was extracted in the tube by adding 20 μl of 50 m*M* NaCl, 20 m*M* tris, *p*H 7.5, and 25 m*M* EDTA. After digestion in 1% SDS and proteinase K (200 μg/ml) for 16 hours at 37°C in the dark, the sample was extracted with phenol (1×), phenol/chloroform (2×), and then chloroform (2×). Extracted DNA was ethanol-precipitated and then suspended in 200 μl of TE. DNA from liquid cultures of Charon 21A was prepared (*16*), digested with Hind III to completion, and dephosphorylated with calf intestinal alkaline phosphatase (BRL). Test ligation and infection of LE392 confirmed a background of $\leq 10^4$. Flow-sorted CH19 DNA was cut to completion with Hind III, phenol-extracted, and precipitated with 2.5 volumes of ethanol and 0.5*M* NaCl. Ligations (*18*) were performed at 14°C for 16 hours in 10 m*M* tris-Cl, *p*H 7.5, 5 m*M* $MgCl_2$, 1 m*M* ATP, gelatin (50 μg/ml), and T4 DNA ligase (100 U/ml; N. E. Biolabs) at a DNA concentration of 300 μg/ml. Low-density plating of the packaged DNA on minimal plates and screening with an Alu repeat clone, Blur2 (*19*), was performed as described (*4*) and with the substitution of Colony Plaque Life Membrane (NEN-DuPont) for the nitrocellulose. Small quantities of DNA from plaques that did not hybridize were prepared by liquid lysis (*20*), Hind III–digested, separated by electrophoresis, blotted, and rescreened by means of nick-translated total human DNA or Blur2 probes. Hind III insert fragments that demonstrated null hybridization to human repeat DNA were selected for further study. Inserts of interests were subcloned into an pSP64 (ProMega). Inserts were restriction-mapped by single or multiple digests with the suppliers' recommended buffers.

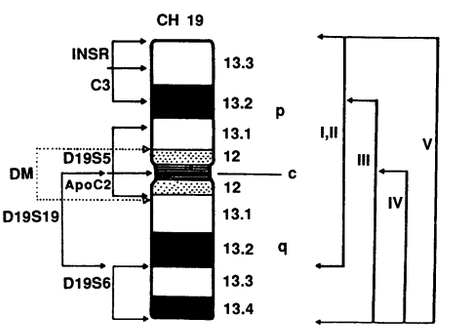

Fig. 3. A panel of human-rodent hybrid cells containing portions of CH19 were used to regionally map clones. Cell types with the portion of CH19 present are as follows: (I) RAG hybrid cell line with t[19 pter → q13.3::Xq23 → qter]; (II) A9 hybrid cell lines with t[19pter → q13.3::Xq23 → qter]; (III) RAG hybrid cell lines with t[Xqter → ql::19p13.2 → qter]; (IV) B82 hybrid cells with t[1pter → 1cen::19cen → qter]; and (V) normal male and female lymphoblast cells. The localization of loci indicated to the left was as follows: INSR (insulin receptor) was by in situ hybridization (*21*) to 19 pter → 19 p13.2 and hybridizes to cell types I, II, and V; C3 (complement C3) (*22*) was mapped by in situ hybridization and hybrid cells to the distal portion of 19p13.2 → 19p13.3 (*23*) and hybridizes to cell types I, II, and V; D19S5 and D19S6 by in situ hybridization and hybrid cells (*23*) to 19p12 and to 19q13.3 → 19qter, respectively, and hybridizes to cell types I, II, III, and V and to III, IV, and V, respectively; ApoC2 (apolipoprotein C2) (*24*) and D19S19 (LDR152) hybridized to all chromosome 19–containing cell types. Cells that contained the q-terminal portion of the human X chromosome translocated to CH19 were grown on HAT (hypoxanthine, aminopterin, thymidine) medium (Gibco) to select for the HGPRT (hypoxanthine-guanine phosphoribosyl transferase) gene. The 1::19 translocation lines were grown briefly on Dulbecco's modified Eagle's medium to extract DNA, and determination of the presence of the long arm of CH19 was with two probes with confirmed long-arm localization. Separate blots from all cell lines were constructed from digests by means of Hind III, Taq I, Msp I, or Bam HI.

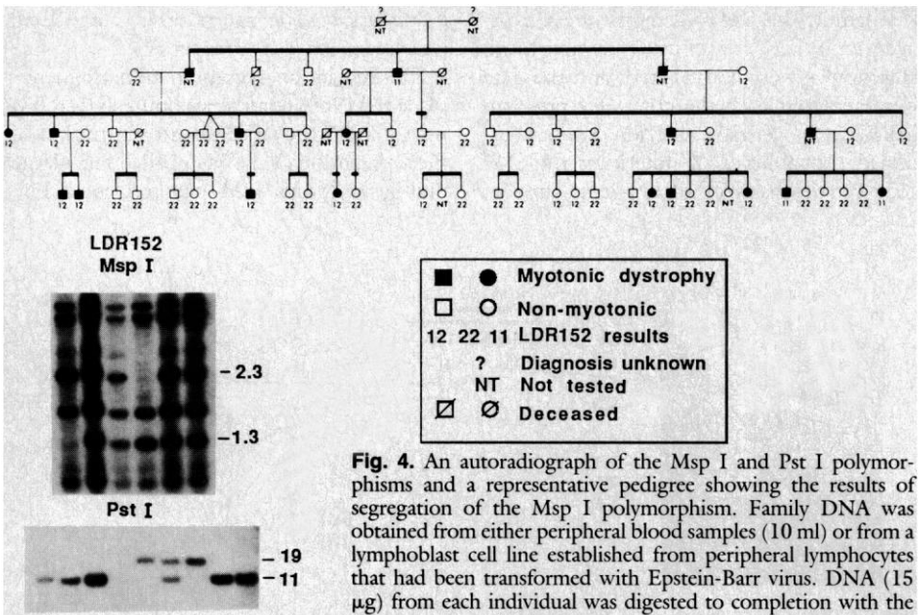

Fig. 4. An autoradiograph of the Msp I and Pst I polymorphisms and a representative pedigree showing the results of segregation of the Msp I polymorphism. Family DNA was obtained from either peripheral blood samples (10 ml) or from a lymphoblast cell line established from peripheral lymphocytes that had been transformed with Epstein-Barr virus. DNA (15 μg) from each individual was digested to completion with the restriction enzymes for 3 to 5 hours. A second digestion was performed after phenol extraction and ethanol precipitation. Digested DNA samples were loaded onto 1% agarose gels in TAE (*20*), and electrophoresis was for 16 hours at 25 mA constant current. Samples were blotted onto GeneScreen Plus (NEN-DuPont). LDR152 was subcloned into the Hind III site of pSP64. Labeled probe was prepared by transcription of the linearized plasmid with SP6 polymerase (ProMega) and ^{32}P-UTP (NEN). Hybridization was at 42°C in 50% deionized formamide, 10% dextran sulfate, 1% SDS, 20 m*M* sodium phosphate (*p*H 7.0), 1*M* NaCl, 5× Denhardt's, and sheared salmon sperm DNA (100 μg/ml). Filters were washed according to the supplier's recommendation except with an additional wash in 0.1× standard saline citrate, 0.1% SDS at 65°C. Autoradiography was at −70°C with a DuPont Cronex intensifying screen for 1 to 2 days. In the pedigree, the 2.3-kb fragment was designated as allele 1; the 1.3-kb allele was designated as allele 2.

4B. The 2.3-kb allele appears to be linked to DM in this family. Previous analysis of the six DM pedigrees with the polymorphic probes for the ApoC2 locus detected a single crossover event (*2*) which is not yet informative for D19S19.

New RFLPs developed by chromosome walking should make D19S19 more informative and help to delineate the order for the three loci: DM, ApoC2, and D19S19. Increased heterozygosity for D19S19 will also prove useful for carrier detection and prenatal diagnosis. Should the three-point order indicate that the ApoC2 and D19S19 loci flank DM, then the error in carrier detection and prenatal diagnosis with the combined use of these two probes will be <<1%. Flanking markers will also aid in the isolation of the DM gene by chromosome walking and/or hopping methodologies (*8–12*). The relationship of recombination frequency and physical distance has not been determined for the human CH19 centromeric region. Macro-restriction mapping (*13*) with rare cutting endonucleases combined with pulsed field gel electrophoresis (*9–12*) will provide a method for correlating probe distances with recombination frequencies.

LDR152 and ApoC2 are both useful in gene carrier diagnosis in DM. DM may not be diagnosed clinically until after the childbearing years in a significant proportion of cases. The major clinical application thus far has been to identify those individuals at risk who do not carry the gene and allow them to proceed with families with the knowledge that there is less than a 3% chance that they will be carriers. The ethical dilemma in the diagnosis of Huntington's disease is not a practical problem for DM patients since diagnosis of a presymptomatic DM individual does not predict a rapid, lethal disease. Since cardiac complications of DM can frequently be treated, presymptomatic carrier detection will permit early intervention in cardiac manifestations (*14*). The most severe form of DM, congenital DM, only occurs in 10 to 20% of the affected children of female gene carriers. It is now possible to provide accurate prenatal diagnosis of congenital DM in affected women with a combination of molecular genetic diagnosis and ultrasonography.

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17. Cultured cells used to prepare metaphase chromosomes were from VL324, a normal female lymphoblast line established in this laboratory. Cultures were maintained in RPMI 1640 + 15% fetal bovine serum for 3 to 4 days. For harvesting metaphase cells, flasks containing 150 ml of suspended cells (1.5×10^8) were supplemented with 50 ml of additional fresh media + 4×10^{-7}*M* methotrexate (Lederle) to block cells in S phase. After 21 hours, cells were released by washing with unsupplemented RPMI 1640 and then resuspended in one-half volume of growth media containing 10^{-5}*M* thymidine (Sigma) and Colcemid (0.09 μg/ml; Gibco). Eight hours later cells were pelleted in a Sorval GSA rotor (1500 rev/min, for 10 minutes). Cells were pooled in 50 ml of a modified hypotonic buffer composed of 10 m*M* sodium citrate, *p*H 7.6, 50 m*M* KCl, 10 m*M* $MgSO_4$, 3 m*M* dithiothreitol, and Colcemid (0.09 μg/ml), with gentle shaking for 20 minutes. Cells were then pelleted in a clinical centrifuge, and buffer was removed. Mitotic indices were generally 40 to 50% with this protocol. After vortexing cells into suspension, 0.6 ml of 2% Triton X-100 and 0.6 ml of glacial acetic acid were added, and the suspension was again vortexed to lyse cells. Chromosomes were released by passing the suspension through a 22-gauge needle (*16*). By means of a modification of the method of Stubblefield and Wray (*16*), metaphase chromosomes were fractioned on 30 to 55% sucrose gradients buffered in hypotonic buffer in the absence of acetic acid. Rate zonal linear sucrose gradients of 36 ml were poured in six incremental 6-ml steps (55, 50, 45, 40, 35, and 30%) and allowed to diffuse to approximate linearity at room temperature for 8 to 12 hours prior to centrifugation. Aliquots (2.5 ml) of chromosome suspension in 10% acetic acid and 0.2% Triton X = 100 were layered onto each gradient and centrifuged at 3100 rev/min for 30 minutes at 25°C in a Sorval HB-4 rotor. Fractions containing 1 ml were dripped by capillary siphoning from the bottom of the tubes with the apertures of the capillary positioned 5 to 7 mm above the interphase nuclear pellets.
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