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Physical Localization of Two DNA Markers Closely Linked to the Cystic Fibrosis Locus by Pulsed-Field Gel Electrophoresis

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Summary

Our previous linkage analysis suggested that the DNA segment D7S122 is located between MET and D7S8, the two genetic markers that are thought to flank the cystic fibrosis locus (CF). Subsequent chromosome walking experiments revealed that D7S122 is within close distance to another randomly isolated DNA marker, D7S340. To determine the physical relationship among D7S122, D7S340, MET, and D7S8, we have constructed a long-range restriction map of the region containing these four DNA segments, by using DNA from a human/hamster somatic hybrid cell line 4AF-KO15 (containing a single human chromosome 7) and a series of rare-cutting restriction enzymes. The combined results of complete, partial, and double digestion analyses confirm that D7S122 and D7S340 are located between MET and D7S8. The order of these markers is MET-D7S340-D7S122-D7S8, with distance intervals of approximately 500, 10, and 980 kbp, respectively. Together with family analysis, this information will be useful for eventual identification of the CF gene.

Introduction

Cystic fibrosis (CF) is a common autosomal recessive disorder, with an estimated gene frequency of $\sim 0.02$ in the Caucasian population in North America (Boat et al. 1989). The basic defect of this disease is unknown, but the disease locus (CF) has been confined to a small region on the long arm of chromosome 7, band q31-q32, between MET and D7S8, through a series of extensive genetic linkage and physical mapping studies (reviewed in Tsui et al. 1989). Recent family studies have further narrowed the location of CF to a region between D7S23 and D7S8 (Farrall et al. 1988). The physical distance between MET and D7S8 has been estimated to be 1.5–2 million base pairs (mb) (Drumm et al. 1988; Poustka et al. 1988), and that between D7S23 and D7S8 has been estimated to be 700–1,200 kb (Poustka et al. 1988).

To facilitate isolation of the CF gene on the basis of its chromosome localization, additional DNA markers useful for CF mapping were isolated from a flow-sorted chromosome 7-specific genomic library (Rommens et al. 1988). This effort resulted in the isolation of 258 DNA segments, 53 of which were from the region of interest. Genetic linkage studies were performed with a subset of these markers that revealed RFLPs. One of these markers, D7S122, was found to be located between MET and D7S8 and tightly linked to CF (Rommens et al. 1988). In addition, long-range physical mapping studies were initiated for all the q31-q32 markers, by pulsed-field gel electrophoresis. The results of these studies revealed that D7S122 shared a large number of restriction fragments with another DNA segment, D7S340, when high-molecular-weight genomic DNA was digested with several rare-cutting restriction enzymes (Rommens et al. 1988). Subsequent chromosome walking experiments showed that these two DNA segments were in fact only 8 kb apart (Rommens et al. 1989). Their relative position with respect to other CF-linked DNA markers (i.e., MET, D7S8, and D7S23) was, however, not immediately clear.
To determine the physical location of D7S122 and D7S340, we have performed detailed long-range restriction mapping of the chromosome region containing these two DNA segments and MET and D7S8. Since no rare-cutting restriction-enzyme recognition sites were found between D7S340 (TM58) and D7S122 (pH131) (Rommens et al. 1988), the two markers were used interchangeably as probes in hybridization analysis of the pulsed-field gels. To avoid restriction-site polymorphisms and variations in methylation patterns that would interfere with recognition of the rare-cutting enzymes, the single human chromosome 7 contained in the somatic cell hybrid 4AF/102, subclone KO15 (Arfin et al. 1983; Rommens et al. 1988), was chosen to generate a primary map of the region. Supportive evidence and additional mapping information were obtained from analysis of a diploid human lymphoblastoid line HSC55 (Rommens et al. 1988). Data are presented in this report to confirm that D7S340/D7S122 physically maps between MET and D7S8 and to show that the respective intervals are ~500 kb and ~980 kb.

Material and Methods

Preparation of High-Molecular-Weight Genomic DNA

The DNA samples for pulsed-field gel electrophoresis were prepared from cultured cells embedded in low-melting-point (LMP) agarose (Seakem) essentially according to methods described elsewhere (Gardiner et al. 1986; van Ommen and Verkerk 1986). The human/hamster somatic cell hybrid 4AF/102/KO15, containing a single human chromosome 7 as its only human material, was maintained in α-MEM (minus asparagine) supplemented with 10% FCS. The monolayer cells were harvested at semiconfluence by detaching them from culture dishes with SE buffer (75 mM NaCl, 25 mM EDTA, pH 8). The human lymphoblastoid cells HSC55 were grown in RPMI-1640 medium with 10% FCS; the cell clumps were dispersed by repeated pipetting 12–16 h prior to harvest. The harvested cells were collected by centrifugation at 2,000 g, were washed with additional SE buffer, and were resuspended in fresh SE buffer to a final concentration of 1–4 × 10⁷ cells/ml. The cell suspensions were warmed to 37°C, were quickly mixed with equal volumes of 1% molten LMP agarose (Ultra-pure; Bethesda Research Labs), and were poured into square petri dishes to a thickness of approximately 1.5 mm. The solidified cell suspensions were cut into square blocks (5 mm × 5 mm × 1.5 mm) with a clean scalpel, were transferred to 10 vol lysis buffer (0.25 M EDTA, 1% lauryl sarcosyl, pH 8), and were treated with 0.5 mg proteinase K (Boehringer/Mannheim)/ml at 50°C overnight with gentle shaking. The deproteinized blocks were then washed extensively with several changes of large volumes of TE (10 mM Tris-HCl, 10 mM EDTA, pH 8) at 37°C with gentle shaking and were stored at 4°C ready for restriction-enzyme digestion.

Restriction-Enzyme Digestion

The agarose blocks containing high-molecularweight DNA were washed briefly in sterile double-distilled water and equilibrated overnight in 4–6 vol buffers appropriate for various restriction-enzyme digests, except that BSA and reducing agents were not included. Restriction digestions were then performed with 1–5 units enzyme/µg DNA in 3 vol fresh buffers with BSA and reducing agents for 2–6 h under conditions recommended by the suppliers (New England Biolabs and Boehringer/Mannheim). The reactions were terminated with the addition of EDTA (pH 8) to a final concentration of 10 mM, and the blocks containing digested DNA were stored in pulsed-field gel running buffer (see below) at 4°C ready for electrophoresis.

Pulsed-Field Gel Electrophoresis

DNA samples digested by various rare-cutting restriction enzymes were fractionated by electrophoresis in a cross-field gel apparatus according to a method described by Southern et al. (1987). Generally, 1–2 µg digested genomic DNA (i.e., 1/4–1/2 of the agarose block as described above) were used per each sample well in a 1% agarose (Seakem) circular gel. This amount of DNA used per lane was crucial in order to ensure reproducibility and accuracy in sizing restriction fragments. For enzymes that tend to produce DNA fragments of similar sizes (e.g., XhoI), partial digestions were performed to reduce band-migration distortion due to high local DNA concentration.

Electrophoresis was carried out with a constant electric field of 6.5–8 V/cm in 0.5 × TBE (45 mM Tris-borate-EDTA, pH 8.3) running buffer, and the gel switched between two positions with a total rotation angle of 120°C at time intervals regulated by a personal computer. The duration of electrophoresis at each position (pulse) increased linearly with time; the initial and final pulses were set as indicated. The temperature of the running buffer was maintained at 12°C–14°C with the use of a cooling coil linked to a refrigeration unit.

DNA Size Standards

Oligomerized λ phage DNA and yeast chromosomes
were used as markers for the estimation of DNA fragment sizes. Phage particles were prepared by heat induction of a lysogen Escherichia coli W3110 (λcI857S7) according to standard procedures (Maniatis et al. 1982) and were suspended in 0.5% LMP agarose to yield a concentration of 50–100 μg DNA/ml. Phage capsids were removed by sarcosyl and proteinase K treatment as described above for mammalian cells. Oligomers of as large as 25 unit-length (48.5 kb) λ DNA annealed at their cohesive ends could be observed. Chromosomal DNA from Saccharomyces cerevisiae strain SC252 (gift from J. D. Friesen) was prepared according to a method described elsewhere (Carle and Olsen 1985). The sizes of the yeast chromosomes were calibrated with the λ oligomer ladder.

DNA Probes

The majority of DNA probes used in the present study have been described elsewhere: metD (White et al. 1986), metH (White et al. 1985), pJ3.11 (Wainwright et al. 1985), p3H-1 (Dean et al. 1987), and pH131 and TM58 (Rommens et al. 1988). The two latter probes were found to be 8 kb apart (see text); however, TM58 was used for most of the experiments, because it gave a better hybridization signal when used as a probe in gel blot hybridization analysis. Two additional DNA segments (W3D1.4 and EGI.4) derived from chromosome walking were also used in the present analysis. They are ~20 and ~60 kb from pH131 toward D7S8, respectively. Details of W3D1.4 and EGI.4 have been published elsewhere (Rommens et al. 1989).

DNA segments were purified from plasmid vectors according to a method described elsewhere (Rommens et al. 1988) and were labeled with [α-32P]-dCTP as hybridization probes by the random-priming method (Feinberg and Vogelstein 1983) to a specific activity of 4–10 x 10⁶ cpm/μg DNA.

Hybridization Analysis

The DNA samples in the agarose gels were visualized by staining with ethidium bromide after electrophoresis. To facilitate subsequent blot transfer, the DNA fragments were depurinated by treating the gel with 0.25 N HCl for 10 min. Following denaturation in 0.4 N NaOH/0.6 M NaCl and neutralization in 0.5 M Tris-HCl (pH 7.5)/1.5 M NaCl for 30 min each, the DNA samples were transferred to either ZetaProbe® (Bio-Rad) or NYTRAN® (Schleicher and Schuell) membranes according to the method of Southern (1975). Prehybridization treatment and hybridization with radioactively labeled DNA probes were carried out with the procedure of Church and Gilbert (1984). Hybridization was performed at 65°C–68°C, and washing of the membranes was carried out at the same temperatures with 0.1–0.2 x SSC (1 x SSC = 150 mM NaCl, 15 mM Na citrate) and 0.1% SDS. Autoradiography was performed with Kodak X-OMAT AR film in the presence of Dupont Lightning Plus intensifying screen for 1–5 d. Membranes were reused for hybridization with other DNA probes after deprobing with 0.4 M NaOH and neutralization with 0.2 M Tris-HCl (pH 7.5), 0.5% SDS, 0.1 SSC, each procedure being done at 42°C for 30 min.

Results

Linkage between D7S122/D7S340 and MET

The first set of mapping data was obtained from hybridization analyses of the somatic cell hybrid 4A/E/102/KO15 DNA with the metD and TM58 probes. Both MET (metD) and D7S122/D7S340 (TM58) were found to be located on a 580-kb NrtI fragment but to be separated by a single NolI site, such that, in a NolI and NrtI double digestion, a 360-kb fragment was detected with TM58 and a 220-kb fragment with the metD probe (fig. 1A). Single restriction recognition sites for BssHII and MluI were also found between the two loci (see below). In addition, the metD and TM58 probes were found to hybridize to two different XhoI bands, 250 and 300 kb, respectively, but to the same 550-kb partial digestion product (fig. 1B), suggesting that metD and TM58 were in close proximity to each other and that the maximal distance between them was less than 550 kb. The results of SfiI complete and partial digestions, however, did not show any overlapping fragments between metD and TM58 (fig. 2). A tentative map could also be constructed on the basis of the Saff complete and partial digestion data, indicating that metD and TM58 were ~300 kb apart (fig. 3). Furthermore, a Sall site was found between met H, the 3' most exon of the met gene (Dean et al. 1987), and metD, placing TM58 downstream (to the 3' side) of the MET locus.

Additional mapping data could be obtained with probes derived from the chromosome walking experiments (Rommens et al. 1989). Since three XhoI sites were identified within 50 kb of the starting sites D7S122 (pH31) and TM58, it was possible to map these two loci with complete and partial digestions with XhoI. The resolution of the pulsed-field gel around the 20–50-
kb region was poor, but the data clearly showed that at least two of the potential sites in the cell line KO15 were susceptible to XhoI digestion. As shown in figure 1C, the probe W3D1.4 detected a 10-kb XhoI fragment and a number of other bands which were consistent with being the products of partial digestions at the indicated XhoI sites. This result thus oriented pH131 and TM58 with respect to MET, providing important information for devising subsequent chromosome walking and jumping strategies.

**Linkage between D7S122/D7S340 and D7S8**

Our chromosome walking experiment also revealed an NruI site at ~80 kb from TM58 (Rommens et al. 1989). As expected, a different NruI fragment was detected with the probe EGL4 obtained from the other side of this site (see fig. 4). More important, however, the D7S8 locus (3H-1) was also found on this 1.6-mb NruI fragment, suggesting a maximal distance of 1.7 mb from TM58. On a separate gel, a partial NruI digestion product (~2.2 mb) hybridizing to both TM58 and
J3.11 (another probe for D7S8) could be detected (data not shown). These observations thus confirmed the order of MET, D7S22, and D7S8 that was derived from the genetic linkage data (Rommens et al. 1988).

As most of the common restriction fragments detected by TM58 and J3.11 in the KO15 cell line were large, it has been difficult to obtain an accurate estimate of the distance between the two; for example, the NotI fragment was ~5 mb, MluI >5 mb, and BssHII >2 mb (data not shown). Similar results were obtained with DNA from other cell types (Drummond et al. 1988; Poustka et al. 1988; Fulton et al. 1989; authors’ unpublished data), suggesting that the region is generally deficient of recognition sites for these enzymes. After testing additional enzymes, however, we found that it was possible to use partial NaeI digestions with the KO15 cell line to obtain the required information. This enzyme produced a 460-kb fragment for metH, with partial digestion products of 550, 680, and 770 kb (fig. 5). When the same blot was hybridized with pH131, W3D1.4, or EG1.4, a 680-kb NaeI fragment and a partial digestion product of 1,280 kb were observed; no obvious overlapping fragments with the met probe were found. When probed with 3H-I (D7S8), however, fragments of 20, 220, and 620 kb were detected, suggesting the presence of a 600-kb NaeI fragment between TM58 and D7S8 (fig. 5). Furthermore, when the J3.11 probe (D7S8) was used, bands of 200, 220, and 600
kb in size were observed (data not shown), consistent with 3H-1 and J3.11 being only 10 kb apart (Dean et al. 1987) and separated by a Nael site. A more exact distance between TM58 and D7S8 could be derived from Nael and Nrdl double digestion, which produced a 360-kb fragment for TM58 and a 300-kb fragment for EG1.4 (data not shown). In conclusion, we estimated the distance between TM58 and D7S8 to be ~980 kb (see fig. 6).

**Figure 3**  *Sall* analysis. DNA samples were prepared as described in the legend to fig. 1, except that the gel was run at 7 V/cm for 20 h and a ramped switching time of 10–40 s. The same blot (two lanes) was hybridized to the metD, metH, and W3D1.4 probes.

**Figure 4**  *Nrdl* analysis. DNA samples were prepared as described in the legend to fig. 1, except that the gel was run at 8 V/cm for 20 h and a ramped switching time of 5–150 s. The same blot (three lanes) was hybridized to the metH, TM58, pHi131, EG1.4, and 3H-1 probes.
Figure 5  NaeI analysis. DNA samples were prepared according to procedures described in Material and Methods. The electrophoresis conditions were identical to those in fig. 4.

Alignment of Restriction Sites

The alignments of the various restriction sites were achieved by double digestions and by comparisons with the short-range restriction map derived from overlapping, cloned DNA segments (Rommens et al. 1989). The NruI sites were used as reference points for most of the other enzymes, as has been outlined for NaeI and NotI. The BssHII site within the 580-kb NruI fragment was aligned by the presence of both a 370-kb BssHII-NruI fragment detected with TM58 and a 210-kb fragment with the metD probe (data not shown). Similarly, the MluI site was positioned by the presence of both a 450-kb MluI-NruI fragment detected with TM58 and a 130-kb fragment with metD. The positioning of the XhoI and SalI sites to the NruI site near D7S122/D7S340 was primarily achieved by chromosome walking. The SalI site adjacent to the EG1.4 probe was first predicted by the presence of a 40-kb SalI and BssHII double-digestion product (data not shown) and was subsequently confirmed by cloning (Rommens et al. 1989).

Discussion

A summary of the interpretation of our mapping data of the CF region is shown in figure 6. This map has confirmed our previous genetic linkage analysis, which indicated that D7S122 maps between MET and D7S8
Figure 6  Long-range restriction map of the CF region. The CpG-rich island associated with the D7S23 locus (Estivill et al. 1987) is at the NotI site (in parentheses, [ ]) that is not detected in our cell line. DNA fragments sizes derived for individual restriction enzymes are shown on the top, and the composite map is shown on the bottom. A = Nael; B = BstIII; F = Sfil; L = SalI; M = MluI; N = NotI; R = NruI. The XhoI sites are not included in the composite map, to increase clarity. There are additional XhoI, SalI, and Sfil sites in the D7S8 region; they are not shown here, as they are not supported by the presented data. The Sfil site with an asterisk (*) was derived from a Sfil/NruI double digestion probed with J3.II and J3.II; the BstIII, NotI, and NruI sites with parentheses were derived from the cell line HSC55 (data not shown). The BstIII sites with brackets ([ ]) were established by chromosome walking (Rommens et al. 1989).

(Rommens et al. 1988). The presence of fortuitous restriction sites detectable in each of the DNA loci (SalI for MET, NruI for D7S122/D7S340, and Nael for D7S8) in the hybrid cell line used in the present study has allowed precise measurements of distance intervals for the entire region. Since many of the restriction sites deduced from our pulsed-field gel analysis have been confirmed by alignment with cloned DNA segments derived from the chromosome walking and jumping experiments (Rommens et al. 1989), our map probably reflects an accurate estimate of the size of the CF region. Together, the pulsed-field gel analysis and cloning studies have allowed us to determine that the distance intervals between MET, D7S340, D7S122, and D7S8 are 500, 8, and 980 kb, respectively. In addition, these studies have established the orientation of the cloned DNA segments closely linked to the CF locus, providing reference points in chromosome walking and jumping experiments for the purpose of cloning the CF gene.

Although the estimated general distance between MET and D7S8 derived from the present study is in good agreement with those reported elsewhere (Drumm et al. 1988; Poustka et al. 1988; Fulton et al. 1989; Iannuzzi et al. 1989), there are significant variations in the restriction sites spanning these two loci. While the previous maps were based on data obtained from DNA isolated from lymphoblastoid cell lines or peripheral blood cells, the current map is derived from the somatic cell hybrid 4AF/102/KO15. The most notable difference was the resistance to digestion in 4AF/102/KO15 by a variety of rare-cutting restriction enzymes (such as NotI, BssIII, and SalI) at the sites associated with the previously reported DNA marker D7S23 (Estivill et al. 1987; Poustka et al. 1988). As a result, the inclusion of the D7S23 locus in our map was initially difficult. This undermethylated CpG-rich region, located at the 5' end of the IRP locus (Wainwright et al. 1988), was subsequently placed, by fine restriction mapping of recombinant DNA clones isolated by chromo-
some walking and jumping (Rommens et al. 1989), at
~80 kb from D7S122/D7S340 toward D7S8 (see fig.
6). There are, however, some restriction sites that coin-
cide between the KO15 map and that derived from diges-
tion of lymphoblast DNA samples; for example, an
identical 410-kb SfiI fragment, presumably containing
the D7S23 locus, has been detected by TMS8 in both
4AF/102/KO15 and HSC55 (data not shown). In addi-
tion, some of the restriction sites (such as XhoI, SflI,
and NruI) detected by the pulsed-field gel mapping
studies have been confirmed by chromosome walking
using genomic libraries constructed with DNA isolated
from peripheral blood (Rommens et al. 1989).

The overall agreement between our map and those of
others (Drumm et al. 1988; Poustka et al. 1988; Fur-
ton et al. 1989; Iannuzzi et al. 1989) argues against
the possibility of chromosome rearrangement involv-
ing the CF region in 4AF/102/KO15, although chro-
some rearrangement has been observed for other
regions of chromosome 7 in somatic cell hybrids related
to this cell line (authors' unpublished data). The re-
striction-site differences indicated above probably reflect
variations in methylation patterns or DNA sequence
polymorphisms inherent in the genomic DNA used in
these studies. In the latter regard, our mapping data
are consistent with the assumption that the polymorphic
Not1 site detected by the met probes (Collins et al. 1987;
Estivill et al. 1987; Julier and White 1988) is present
in the single human chromosome 7 contained in the
somatic cell hybrid 4AF/102/KO15 (see fig. 6).

A previous study based on family analysis (Farrall et al.
1988) showed that CF is located between DNA
segments KM19 (D7S23) and D7S8, which, according
to our long-range mapping data, are 900 kb apart.
Chromosome jumping studies originated from D7S8
toward MET have allowed the exclusion of a ~200-kb
region as being the location of the CF locus (Iannuzzi
et al. 1989), narrowing the region to be analyzed to
less than 700 kb in size. Approximately 300 kb of this
DNA, extending from D7S23 toward D7S8, has thus
far been isolated by chromosome walking and jumping
from TMS8 (Rommens et al., submitted), and more
than 15 RFLPs have been identified in this region (Kerem
et al., submitted). Studies to determine the relation-
ship of these DNA markers with respect to CF are cur-
rently underway (see Note added in proof).

As a final note, we find that the enzyme XhoI is par-
cularly useful for alignment between the long-range
maps generated with standard rare-cutting enzymes
(such as Not1, SfiI, and BsrHI) and the conventional
restriction maps of cloned DNA with frequent-cutting
enzymes (such as EcoRI, HindIII, and BamHI). First,
the average fragment size for XhoI is ~100 kb, which
is between the scales of the two physical mapping
methods. Second, unlike most other rare-cutting en-
zymes, XhoI does not appear to be seriously affected
by differences in DNA methylation found in most mam-
alian cell lines and tissues, although some sites are
probably more prone to methylation than are others.
While cloning of mammalian DNA in yeast artificial
chromosome vectors is becoming increasingly popular,
XhoI may be considered as one of the key enzymes in
characterization of recombinant clones.

Note added in proof.—We have reported the identifi-
cation of the CF gene after submission of the manu-
script of the present article. The supporting data are
described in three papers (Kerem et al. 1989; Riordan
et al. 1989; Rommens et al. 1989). References to these
papers are included in the present article where appro-
priate. The gene spans ~250 kb and is located within
the 380-kb SflI fragment shown in figure 6. There is
a minor adjustment of fragment sizes for the Nael
digestion near the D7S8 region with the use of additional
DNA probes in further analysis—the fragments of 680
and 600 kb are more accurately estimated to be 690
and 640 kb, respectively.

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