

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 $\sim .02$ 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.

Received June 7, 1989; revision received August 16, 1989.

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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 semiconfluency 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 $1\text{--}4 \times 10^7$ 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 \times 5 mm \times 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-molecular-weight DNA were washed briefly in sterile double-distilled water and equilibrated overnight in 4–6 vol buffers appropriate for various restriction-enzyme digestions, except that BSA and reducing agents were not included. Restriction digestions were then performed with 1–5 units enzyme/ μ g 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 μ g 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., *Xho*I), 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 \times 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° 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(λ C1857S7) according to standard procedures (Maniatis et al. 1982) and were suspended in 0.5% LMP agarose to yield a concentration of 50–100 μ g 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 EG1.4) derived from chromosome walking were also used in the present analysis. They are \sim 20 and \sim 60 kb from pH131 toward D7S8, respectively. Details of W3D1.4 and EG1.4 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^{32} P]-dCTP as hybridization probes by the random-priming method (Feinberg and Vogelstein 1983) to a specific activity of $4\text{--}10 \times 10^8$ cpm/ μ g 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 dephosphorylated 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 \times SSC (1 \times 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 4AF/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 *Nru*I fragment but to be separated by a single *Not*I site, such that, in a *Not*I and *Nru*I 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 *Bss*HII and *Mlu*I were also found between the two loci (see below). In addition, the metD and TM58 probes were found to hybridize to two different *Xho*I 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 *Sfi*I 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 *Sal*I complete and partial digestion data, indicating that metD and TM58 were \sim 300 kb apart (fig. 3). Furthermore, a *Sal*I 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 *Xho*I sites were identified within 50 kb of the starting sites D7S122 (pH131) and TM58, it was possible to map these two loci with complete and partial digestions with *Xho*I. The resolution of the pulsed-field gel around the 20–50-

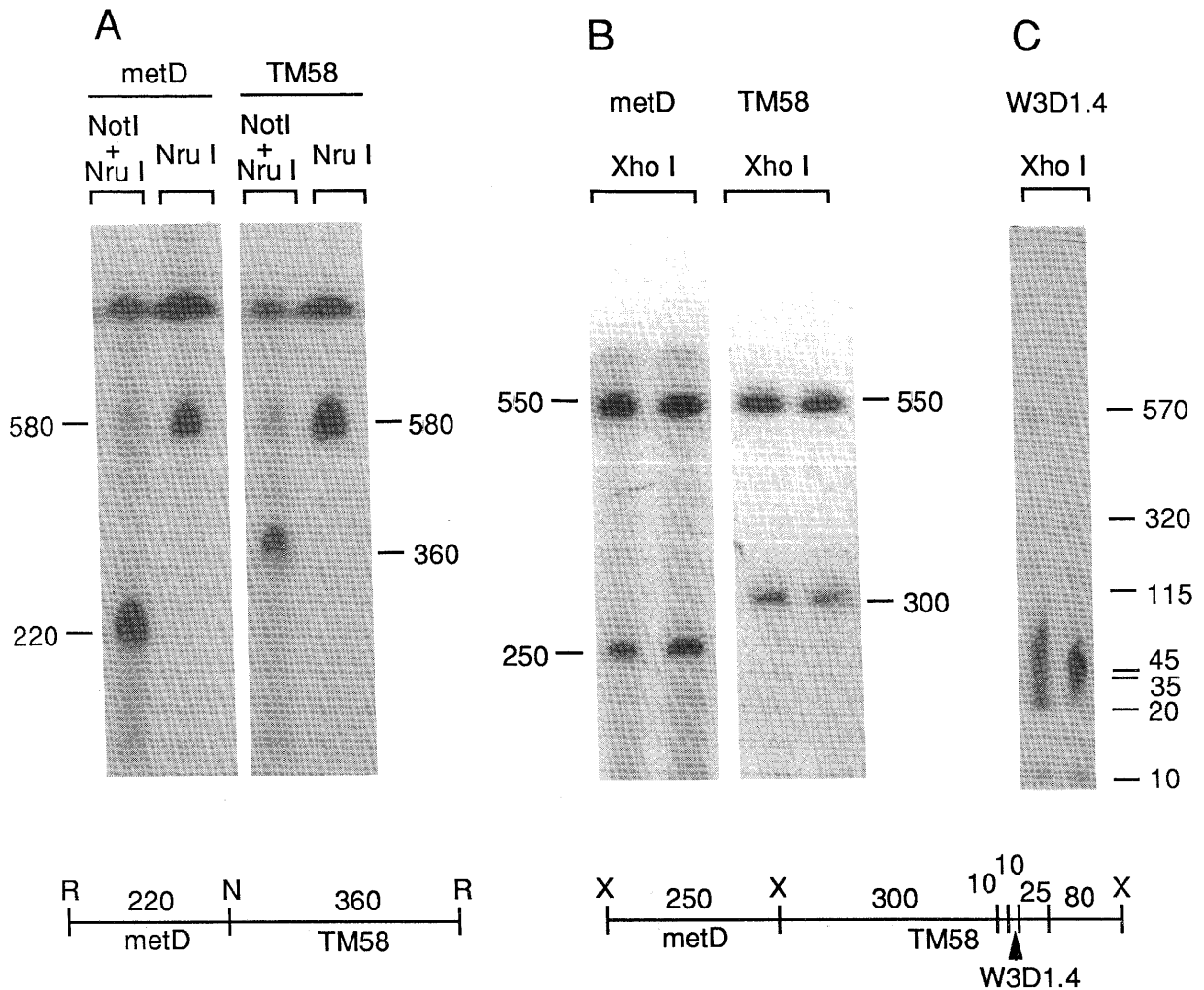


Figure 1 *NotI*, *NruI*, and *XhoI* analyses. DNA samples were prepared from somatic hybrid cell line 4AF/102/KO15 as described in Material and Methods, digested with restriction enzymes as indicated, and separated by pulsed-field gel electrophoresis in a cross-field apparatus with $0.5 \times$ TBE. The running conditions were as follows: A, 8 V/cm for 20 h and a ramped switching time of 15–60 s; B and C, 7 V/cm for 20 h and switching time of 10–40 s. The blots were hybridized with [32 P]-labeled metD, TM58, and W3D1.4 probes as indicated. The double digestion was carried out first with *NotI* and then with *NruI*. The same blots were used for metD and TM58 in panels A and B. Numbers shown are DNA fragment sizes in kilobase pairs, according to bacteriophage λ DNA oligomers and *Saccharomyces cerevisiae* chromosomes. The interpretation of the hybridization results is shown in the schematic maps below. R = *NruI*; N = *NotI*; X = *XhoI*.

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 EG1.4 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

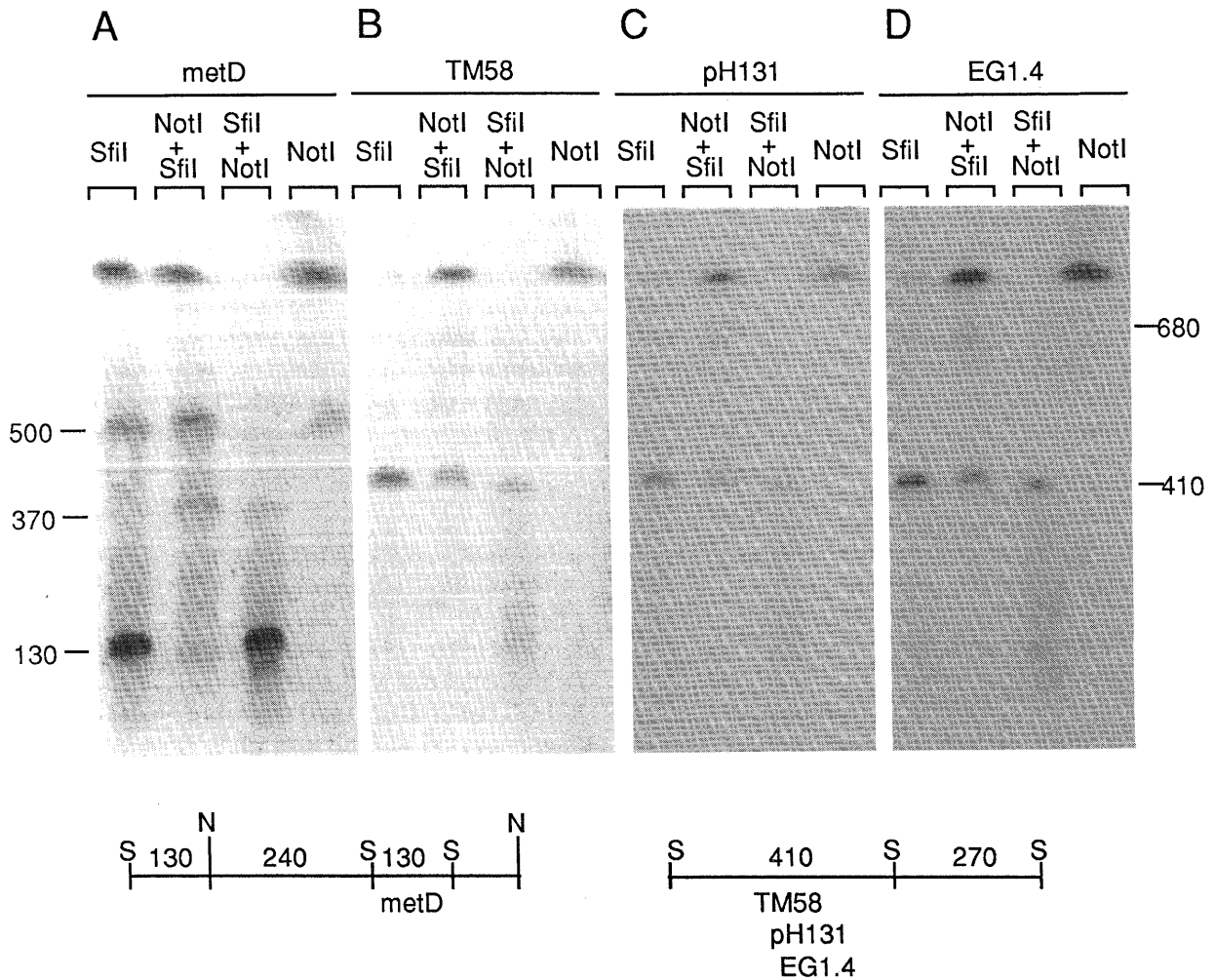
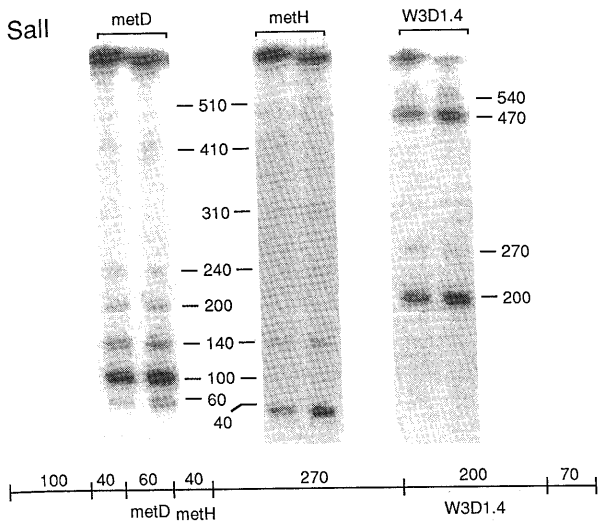


Figure 2 *SfiI* and *NotI* analyses. DNA samples were prepared as described in the legend to fig. 1, except that the gel was run at 6.5 V/cm for 20 h and a ramped switching time of 20–60 s. The same blot was hybridized to (A) metD, (B) TM58, (C) pH131, and (D) EG1.4 probes. The double digestions were performed according to the indicated sequences. The interpretation of the hybridization results is shown in the schematic maps below. Numbers shown are DNA fragment sizes in kilobase pairs. N = *NotI*; S = *SfiI*.

J3.11 (another probe for D7S8) could be detected (data not shown). These observations thus confirmed the order of *MET*, *D7S122*, 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 (Drumm 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 ad-

ditional 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-1 (*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 *NaeI* site. A more exact distance between TM58 and *D7S8* could be derived from *NaeI* and *NruI* 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.

Nru I

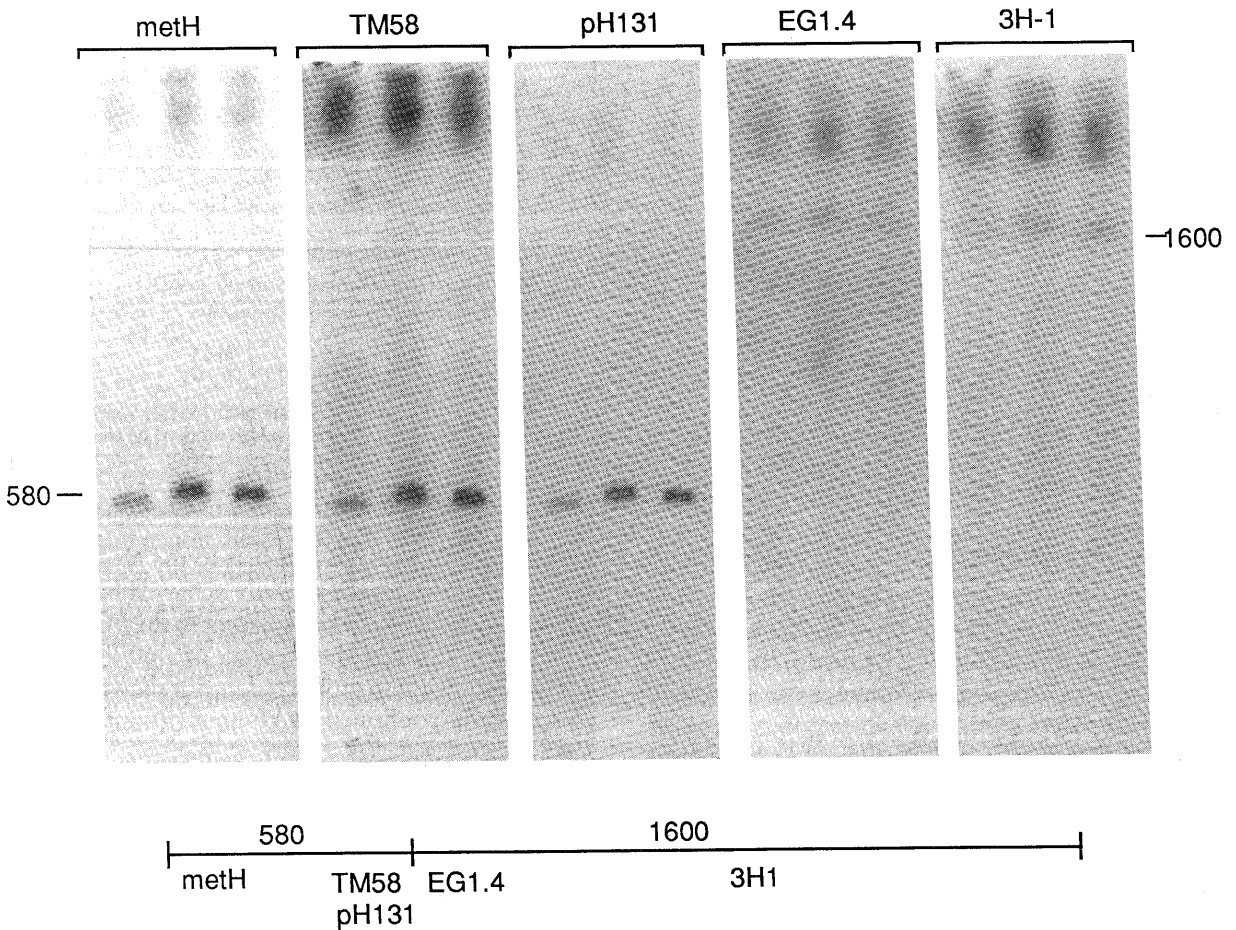


Figure 4 *NruI* analysis. DNA samples were prepared as described in the legend to fig. 1, except that the gel was run at 8 V/cm for 20 h and a ramped switching time of 5–150 s. The same blot (three lanes) was hybridized to the metH, TM58, pH131, EG1.4, and 3H-1 probes.

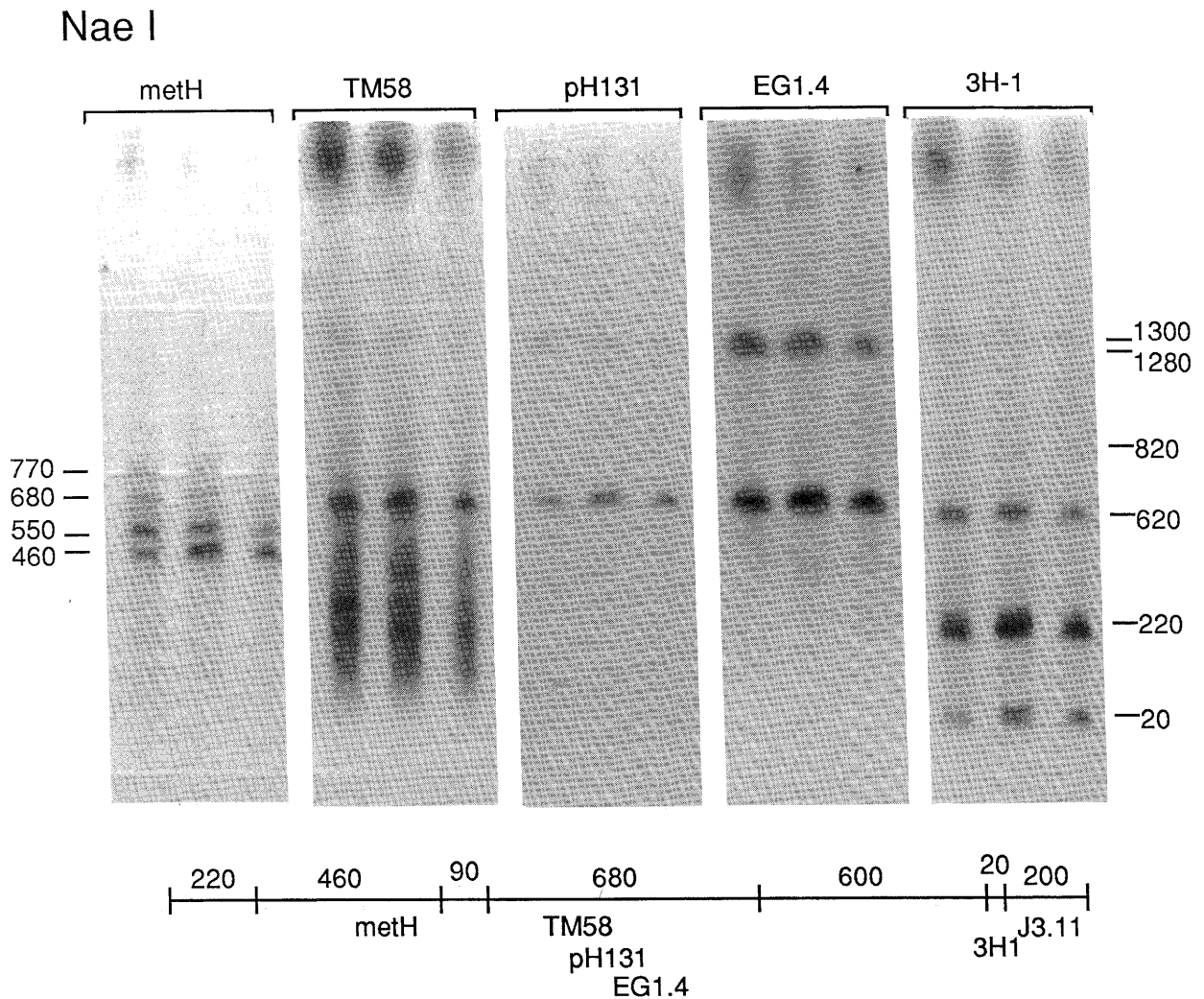


Figure 5 *NaeI* analysis. DNA samples were prepared according to procedures described in Material and Methods. The electrophoresis conditions were identical to those in fig. 4.

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 posi-

tioning of the *XhoI* and *SalI* sites to the *NruI* site near *D7S122/D7S340* was primarily achieved by chromosome walking. The *SfiI* site adjacent to the EG1.4 probe was first predicted by the presence of a 40-kb *SalI* and *SfiI* 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*

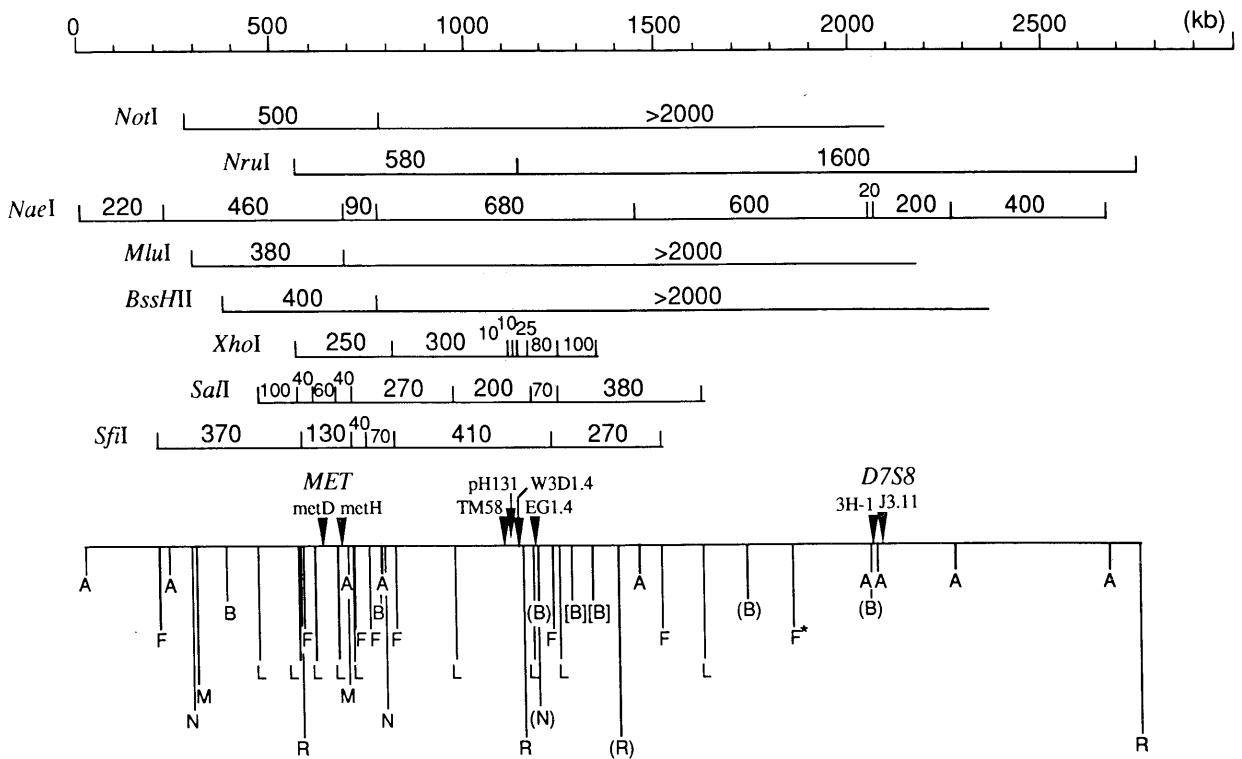


Figure 6 Long-range restriction map of the CF region. The CpG-rich island associated with the D7S23 locus (Estivill et al. 1987) is at the *NotI* site [in parentheses, ()] that is not detected in our cell line. DNA fragments sizes derived for individual restriction enzymes are shown on the top, and the composite map is shown on the bottom. A = *NaeI*; B = *BssHII*; F = *SfiI*; 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 *SfiI* sites in the D7S8 region; they are not shown here, as they are not supported by the presented data. The *SfiI* site with an asterisk (*) was derived from a *SfiI/NruI* double digestion probed with 3H-1 and J3.11; the *BssHII*, *NotI*, and *NruI* sites with parentheses () were derived from the cell line HSC55 (data not shown). The *BssHII* 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 *NaeI* 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*, *BssHII*, and *SacII*) 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 coincide between the KO15 map and that derived from digestion of lymphoblast DNA samples; for example, an identical 410-kb *SfiI* fragment, presumably containing the *D7S23* locus, has been detected by TM58 in both 4AF/102/KO15 and HSC55 (data not shown). In addition, some of the restriction sites (such as *XhoI*, *SalI*, 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; Fulton et al. 1989; Iannuzzi et al. 1989) argues against the possibility of chromosome rearrangement involving the CF region in 4AF/102/KO15, although chromosome rearrangement has been observed for other regions of chromosome 7 in somatic cell hybrids related to this cell line (authors' unpublished data). The restriction-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 *NotI* 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 TM58 (Rommens et al., submitted), and more than 15 RFLPs have been identified in this region (Kerem et al., submitted). Studies to determine the relationship of these DNA markers with respect to *CF* are currently underway (see *Note added in proof*).

As a final note, we find that the enzyme *XhoI* is particularly useful for alignment between the long-range maps generated with standard rare-cutting enzymes (such as *NotI*, *SfiI*, and *BssHIII*) 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 enzymes, *XhoI* does not appear to be seriously affected by differences in DNA methylation found in most mammalian 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 identification of the *CF* gene after submission of the manuscript 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 appropriate. The gene spans ~250 kb and is located within the 380-kb *SalI* fragment shown in figure 6. There is a minor adjustment of fragment sizes for the *NaeI* 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.

Acknowledgments

The authors wish to thank Professor Edwin Southern and Martin Johnson for sharing the original drawing plan of the "cross-field" gel apparatus; Dara Kennedy, Danuta Markiewicz, Natasa Plavsic, and Martha Zsiga for technical assistance; and Francis Collins and members of his laboratory for helpful discussions. This research is part of the Cystic Fibrosis Research Development Program at the Hospital for Sick Children and is supported by grants from the Canadian Cystic Fibrosis Foundation (CCFF), the Cystic Fibrosis Foundation (USA), grant DK-34944 from the National Institutes of Health (USA), and the Sellers Fund. L.-C.T. is a CCFF Research Scholar and Scientist of the Medical Research Council of Canada (MRC); J.M.R. and S.Z.-L. are recipients of a MRC Postdoctoral Fellowship and a predoctoral student award from Deutscher Akademischer Austauschdienst, respectively.

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