RESEARCH

# Positional Candidate Genes for Congenital Chloride Diarrhea Suggested by High-resolution Physical Mapping in Chromosome Region 7q31

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**Congenital chloride diarrhea affects intestinal transportation of electrolytes, resulting in potentially fatal diarrhea. Linkage disequilibrium analyses have suggested the congenital chloride diarrhea gene (CLD) to lie within 0.37 cM from D7S496 in human chromosome 7q31. To clone the CLD gene, we have constructed and refined a physical map based on a 2.7-Mb YAC contig around D7S496 and identified two candidate genes. The physical positions of 4 known genes (DRA, PRKAR2B, LAMB1, DLD), 7 polymorphic repeat markers, and 13 CpG islands were established. DRA (down-regulated in adenoma) is expressed in the gut and encodes a protein with sequence homology to anion transporters, whereas PRKAR2B encodes a regulatory subunit for protein kinase A. Both genes map within 450 kb from D7S496, making them functionally and positionally relevant candidates for CLD.**

Congenital chloride diarrhea [Mendelian Inheritance in Man (MIM) no. 214700; McKusick 1994] is an autosomal recessive disorder of intestinal electrolyte transportation, manifested as a lifetime profuse watery diarrhea with a high chloride content (Norio et al. 1971). The pathogenesis of the disease is unknown, but the defect appears to impair chloride and bicarbonate trafficking in the distal ileum and colon (Turnberg 1971; Holmberg et al. 1975). The diarrhea begins before birth, causing polyhydramnios and often premature birth. Other features include abdominal distention, dehydration, and metabolic alkalosis resulting in death in early infancy if untreated. The treatment includes daily oral replacement of fluid and electrolyte losses. Appropriately treated children grow, develop, and live normally (Holmberg et al. 1977). In Finland, the disease has a relatively high incidence; elsewhere only ~100 cases have been reported since 1945 when the disease was first described (Darrow 1945; Gamble et al. 1945).

The congenital chloride diarrhea gene (CLD) was previously mapped to chromosome 7, close to the cystic fibrosis transmembrane conductance regulator gene (CFTR) (Kere et al. 1993). The high prevalence of CLD in the Finnish population provided an opportunity to refine the genetic localization of CLD by the study of linkage disequilibrium. A combination of haplotype analysis and a quantitative approach ("Luria-Delbrück" analysis; Hästbacka et al. 1992) allowed its fine mapping to 0.37 cM telomeric of D7S496 (Höglund et al. 1995). This genetic distance should be well within the range of physical mapping methods. Thus, we studied overlapping yeast artificial chromosomes (YACs; Burke et al. 1987) from two collections enriched for human chromosome 7 (Scherer et al. 1992; Green et al. 1995) and the Centre d'Etudes du Polymorphisme Humain (CEPH) megaclone collection

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(Cohen et al. 1993). Long-range restriction mapping was used to confirm, refine, and merge a series of distinct YAC contigs of this region and to identify CpG islands as indicators for genes. The previously cloned genes down-regulated in adenoma (DRA) (Schweinfest et al. 1993), PRKAR2B (Solberg et al. 1992), human laminin B1 (LAMB1) (Pikkarainen et al. 1987), dihydrolipoamide dehydrogenase gene (DLD) (Scherer et al. 1991), 13 CpG islands, and 7 polymorphic markers (Weissenbach et al. 1992; Gyapay et al. 1994) were placed on the refined map. As a result, these studies identified two positional candidate genes for CLD and suggest the presence of several uncharacterized genes in and around the candidate region.

## RESULTS

### Identification of Overlapping YACs

To cover the CLD candidate region, 51 YAC clones were identified by systematic sequence-tagged site (STS) content screening from two chromosome 7-enriched collections (Scherer et al. 1992; Green et al. 1995) and the CEPH mega-clone library (Cohen et al. 1993). All screening results were confirmed by PCR assays on DNA extracted from colony-purified clones. STS content mapping (Green and Olson 1990) idenified 14 overlapping segments at D7S496, 8 at D7S501, and 5 at D7S692 (Fig. 1). To close the remaining gap between seed contigs, YAC insert ends were isolated by a ligation-mediated PCR strategy (Kere et al. 1992) and used as probes.

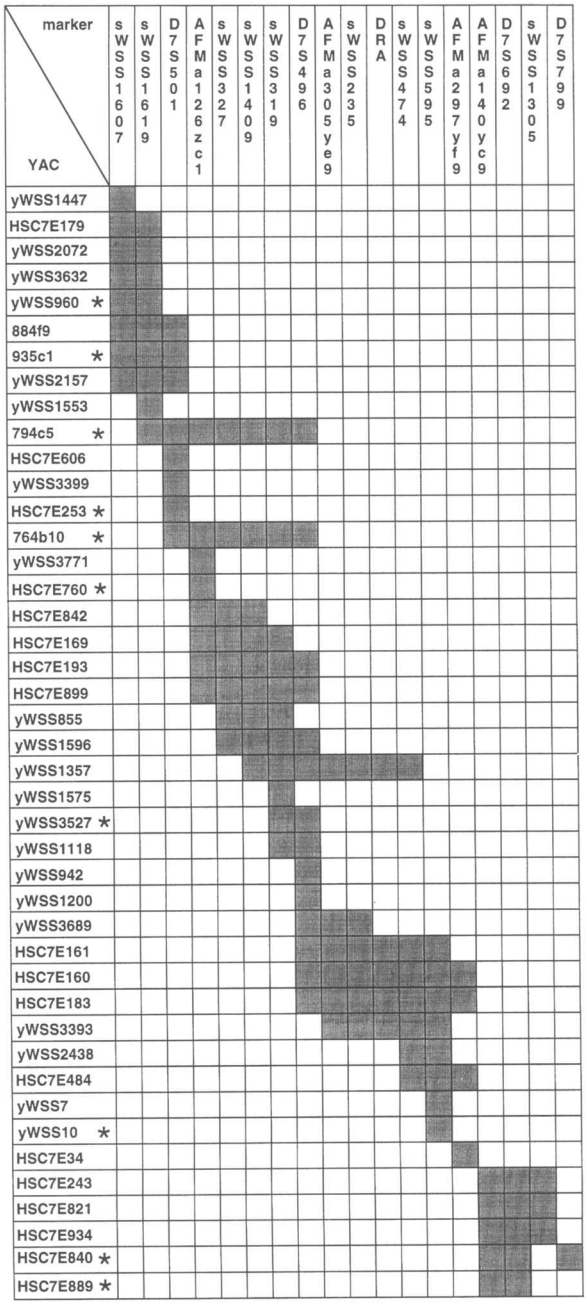


**Figure 1** Anonymous STS and polymorphic marker content of YACs. The shaded boxes denote the extent of each YAC. The YACs marked with an asterisk were discarded from the final physical map (see text). The centromere is to the *left*.

### Rare-cutter Restriction Mapping and Contig Refinement

Altogether 46 YACs were mapped with rare-cutter restriction enzymes. An example of results for two YACs is shown in Figure 2. Restriction maps of tentatively overlapping YACs (see Fig. 1) were compared and used to refine the alignment and orientation of individual YACs. Altogether 13 potential CpG islands were identified based on our definition that a CpG island contains a restriction site for at least two of the four rare-cutter restriction enzymes used.

D7S501 was connected to AFMa126zc1 and D7S496 by STS content mapping of two large clones from the CEPH collection. The respective physical distance was determined by restriction mapping of the clone 884f9 and confirmed by restriction mapping of clones 794c5 and 764b10 using the enzyme *Not*I. On the basis of the sizes of the YACs (both 1000 kb), and the sizes of respective *Not*I fragments, the distance between D7S501 and D7S496 was approximated to be 700 kb.

The final distance map comprises 36 YACs

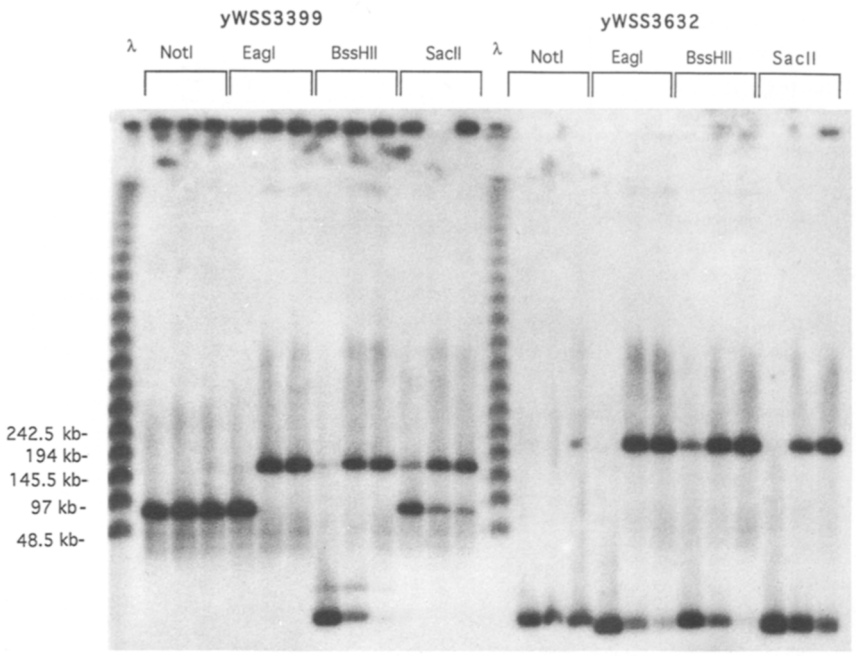


**Figure 2** Restriction mapping of YACs yWSS3399 and yWSS3632 using the pBR322-derived left-arm probe. Each enzyme was used at three different concentrations to obtain serial partial digestions. Bacteriophage λ concatamer was used as a DNA size marker.

and covers 2.7 Mb of DNA. A summary map of the contig is shown in Figure 3. Three clones were discarded because they contained more than one YAC, five because their restriction maps were inconsistent, one because its location remained ambiguous, and one because of an internal deletion. Four small clones were not considered because of dense coverage around their locations.

## Localization of Genes and Markers

The physical order of the STSs and polymorphic markers in the Généthon (Weissenbach et al. 1992; Gyapay et al. 1994) and Cooperative Human Linkage Center (CHLC) (Murray et al. 1994) linkage maps was determined by PCR assays of overlapping YAC clones. The centromere–telomere orientation of the map was based on linkage markers. Table 1 summarizes data for all markers included in the map. Refined mapping of DRA, PRKAR2B, LAMB1, and DLD and markers D7S155 and D7S200 (Rommens et al. 1988) was done by hybridizing specific probes to *Taq*I blots of YACs. The Stanford radiation hybrid panel G3 (Cox 1995) was used to order an additional four markers within and outside the YAC map. The initial screening was performed using PCR markers AFMa288yg1, AFMa126zc1, AFMa297yf9, and D7S799. Positive hybrids were studied with additional markers to define the breakpoints and order adjacent markers. The results are presented in Figure 4. The following order of DNA markers could be determined: 7cen–(AFMb320ve1–AFMa226ye1)–AFMa052ya5–AFMa288yg1–sWSS1607–sWSS1619–D7S501–PRKAR2B–AFMa126zc1–sWSS327–sWSS1409–sWSS319–D7S496–(AFMa305ye9–sWSS235)–DRA–sWSS474–sWSS595–(DLD–LAMB1)–AFMa297yf9–D7S155–D7S200–AFMa140yc9–(D7S692–sWSS1305)–D7S799–D7S523–qter. Physical distances between the markers are shown in Figure 3.

The LAMB1 gene is known to have a CpG island (Vuolteenaho et al. 1990) that contains restriction sites for all enzymes used (*Not*I, *Eag*I, *Bss*HII, *Sac*II). The gene was mapped by hybrid-

**Figure 3** Structure of the YAC contig refined by rare-cutter restriction mapping. Individual YACs are indicated by horizontal lines, drawn to the kilobase scale shown at the bottom. A dot for Cen4 denotes the *left* end of each YAC. The localization of anonymous STSs, polymorphic microsatellites, hybridization probes, and isolated YAC ends are represented by vertical lines. Brackets are used to indicate ambiguity of order. Genes are shown with shaded boxes below the restriction map. Isolated YAC ends are marked with solid boxes in the respective YACs. The restriction maps of each YAC were drawn independently. The experimental variation is thus reflected in that not all sites in YACs are exactly aligned; the consensus map shown in bold below approximates the consensus positions of rare-cutter sites. The centromere is to the *left*. (N) *Not*I; (E) *Eag*I; (B) *Bss*HII; (S) *Sac*II.

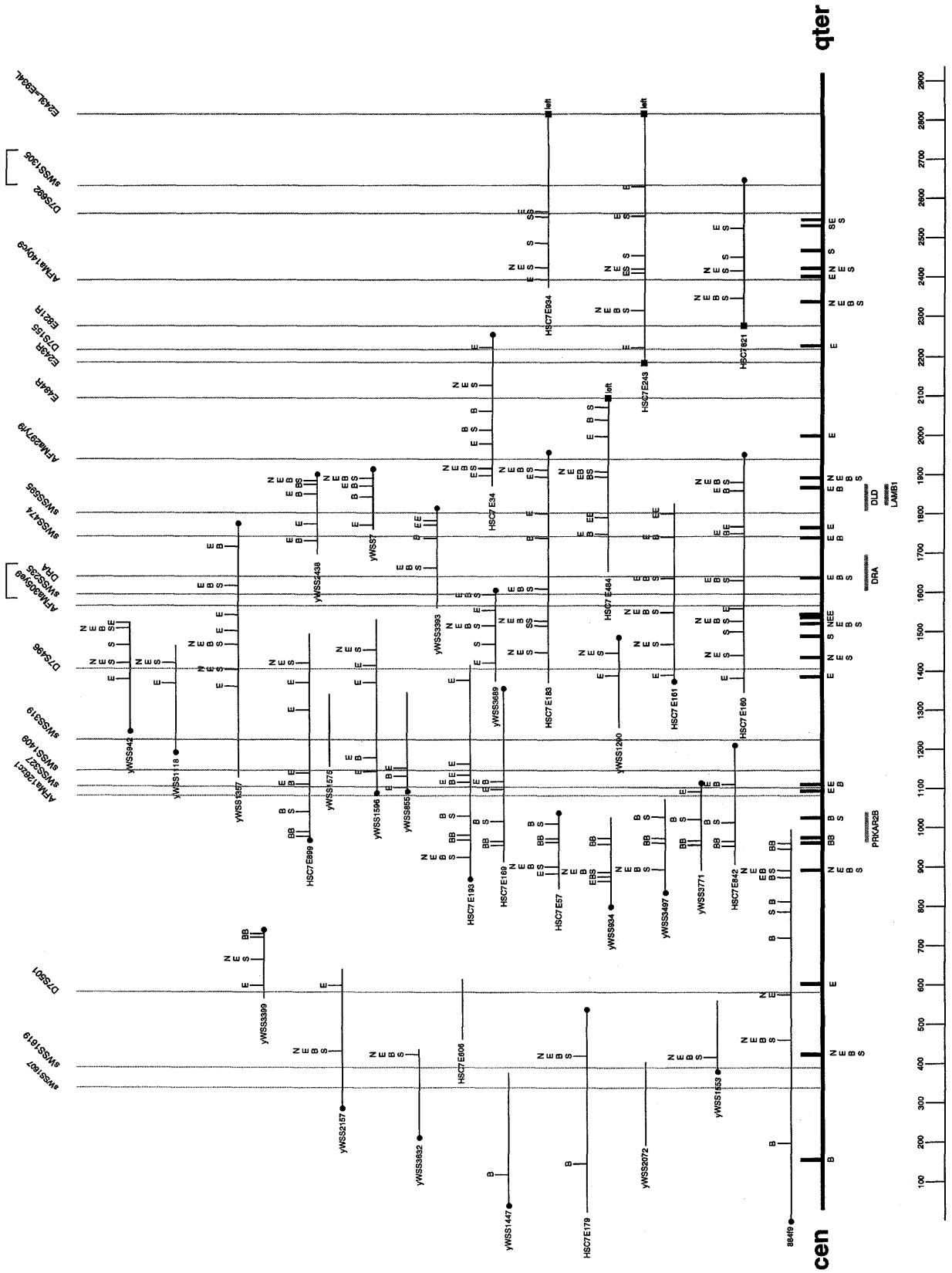


**Figure 3** *(See facing page for legend.)*

## Table 1. PCR Primers and Hybridization Probes Used in This Study

| Marker name | Locus | Primer A | Primer B | Product size (bp) | Reference |
|---|---|---|---|---|---|
| AFM199vb2 | D7S501 | CAC CGT TGT GAT GGC AGA G | ATT TCT TAC CAG GCA GAC TGC T | 163-179 | GDB id: G00-133-374 |
| AFM172xa1 | D7S496 | AAC AAC AGT CAA CCC ACA AT | GCT ATA ACC TCA TAA NAA ACC AAA A | 129-141 | GDB id: G00-133-272 |
| AFM357te1 | D7S692 | CTG ATG ATT GCT ATA GAT ATT CAT C | TGT AAA CAC TTT TGT AGA AGA ACC T | 161-171 | GDB id: G00-141-464 |
| AFMa052ya5 | | TTT AGT ATC TGG NCT TAC GG | ACC GTT AAA CAG CAG TTT CTA | 129 | Genethon unpubl. |
| AFMa226ye1 | | TTT GAG TCT TCA CAG CAG TTG | GGG AGG TTG ATT TCC ACA GT | 200 | Genethon unpubl. |
| AFMb320ve1 | | GGG TCA CCC AAT ATG CTT TC | GGC CTC CCA AAA TGC TG | 250 | Genethon unpubl. |
| AFMa126zc1 | | CCT GTA TGG AGG GCA AAC TA | AAA TAA TGA CTG AGG CTC AAA ACA | 280 | Genethon unpubl. |
| AFMa305ye9 | | AAG AAG TGC ATT GAG ACT CC | CCG CCT TAG TAA AAC CC | 140-150 | Genethon unpubl. |
| AFMa297yf9 | | CTG GAA ATT GAC CTG AAA CCT T | ACA GGG GTC TCT CAC ACA TAT TA | 250 | Genethon unpubl. |
| AFMa140yc9 | | CTA GTC CTG AGA AGA CAT TAC CC | CCT GTT TCA GAT GTT TTA TCC A | 220 | Genethon unpubl. |
| DRAfDRAr | DRA | TTC AAA CTT TGG AAC AAG ATG G | AGT GCC GCC ACT ATA CTG CT | 200 | GDB id: G00-138-165 |
| sWSS1619 | | CCA TTT ACA GAT TGC TTC | TGT GTT TAT GGA GTT CAG | 107 | Genbank n:o G12089 |
| sWSS1607 | | GTA TTT ACT ATT CGG TGG | ATG ACA GTG TGC TTA TGG | 65 | Genbank n:o G00399 |
| sWSS327 | | CTG CAT TCC CCT TCA TTC | GGA CTC GTA AAA TCA CCG | 100 | Genbank n:o L23679 |
| sWSS1409 | | TGT AAA GCC TTA AAC CAG | TTA TGT CAT TTC CCC CTT G | 82 | Genbank n:o G00169 |
| sWSS319 | | CAC TCA CAT ACC ACT CAC | CAC TTG GCT CAA CAC TAC | 82 | Genbank n:o G00009 |
| sWSS235 | | CAA TAA CAA GCA AAG ACT CC | TGT GAC CTG AGT TCT TAG | 127 | Genbank n:o L10595 |
| sWSS474 | | AGG GAT GTA ATT TAC ACA C | AGG CAA ACA TTC TGT ATC TC | 117 | Genbank n:o G00054 |
| sWSS595 | | AGT AAA AGA GGA AAG GAG | TCA CTG TGC CTT GTA AAC | 96 | Genbank n:o G00248 |
| sWSS1305 | | GAT TCA CCA CTG AAA TCT G | CTT CTT TGC CCC CTT AAA TTT G | 60 | Genbank n:o L31277 |

| Probe name | Locus | Fragment size | |
|---|---|---|---|
| pHL-40 | LAMB1 | 0,9 kb PstI fragment | GDB id: G00-119-357 |
| pSP65 | DLD | 1,2 kb EcoRI fragment | GDB id: G00-120-608 |
| hRIIß | PRKAR2B | 3,3 kb EcoRI fragment | GDB id: G00-127-902 |
| E484L | | 250 bp EcoRI-PvuII fragment | This study |
| E243R | | 900 bp EcoRI-AluI fragment | This study |
| E821R | | 650 bp EcoRI-PvuII fragment | This study |
| E243L | | 550 bp EcoRI-PvuII fragment | This study |
| E934L | | 600 bp EcoRI-PvuII fragment | This study |
| p-A6 | D7S155 | | GDB id: G00-124-687 |
| pB-178 | D7S200 | | GDB id: G00-124-736 |

| marker / rad. hybrid number | AFMb320ve1 | AFMa226ye1 | AFMa052ya5 | AFMa288yg1 | sWSS1607 | sWSS1619 | D7S501 | AFM126zc1 | AFMa297yf9 | AFMa140yc9 | D7S692 | sWSS1305 | D7S799 | D7S523 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | + | + | + | + | + | + | + | - | - | - | | | | - |
| 13 | - | - | - | - | - | - | - | + | + | + | + | + | + | - |
| 21 | - | - | - | - | | | | - | + | + | + | + | + | - |
| 33 | + | + | + | + | + | + | + | - | - | - | | | - | |
| 36 | - | - | - | - | | | | - | + | + | + | + | + | + |
| 39 | - | - | - | - | | | | - | + | + | + | + | + | + |
| 44 | + | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 51 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 58 | | | | - | | | | - | - | - | + | + | + | + |
| 64 | - | - | + | + | + | + | + | + | + | + | + | + | + | - |
| 66 | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| 68 | - | - | - | - | | | | - | + | + | + | + | - | |
| 70 | - | - | - | + | + | + | + | + | + | + | + | + | + | - |

**Figure 4** Radiation hybrid panel refines and extends the detailed physical map. Results are shown for clones (vertical) that allowed ordering of markers (horizontal) within and outside the YAC-based map. (+) The hybrids that are positive for a marker studied; (−) the negative hybrids.

ization with a specific probe (pHL-40) in the vicinity of one such island (not shown). These results suggested indirectly a likely orientation for the gene cen–3′–5′–qter.

## DISCUSSION

This study was initiated to aid in the positional cloning of the gene causing congenital chloride diarrhea. The genetic mapping of CLD well within the reach of physical mapping techniques, 0.37 cM from the marker D7S496 and most likely telomeric of it (Höglund et al. 1995) provided a firm basis for this study. Furthermore, no restriction maps of this region were available, and physical relationships were not known between linkage markers and known genes that might be candidates for CLD. Therefore, it was necessary first to construct a high-resolution physical map; we report here the contiguous coverage of 2.7 Mb of DNA that must contain the CLD gene (Höglund et al. 1995).

The initial contigs of YACs were constructed by systematic STS content mapping by identifying clones positive for D7S496, D7S501, D7S692, and their neighboring clones. Gaps were closed by targeted end-fragment isolation. The final physical map was verified by restriction mapping and hybridization with specific probes. The restriction sites between D7S501 and AFMa126zc1 remain ambiguous because of inconsistency in two YACs. However, the distance between these markers is likely to be estimated correctly, based on the same size *Not*I fragment in three mega-YACs. A radiation hybrid panel was used to confirm and refine the order and orientation of an additional three polymorphic markers in relation to the physical and genetic maps, thus providing an opportunity to evaluate these new markers in the haplotype analysis of CLD patients (Fig. 4). The summary restriction map (Fig. 3) was based on consistent sites in at least two overlapping YAC clones.

Four previously cloned genes and nine CpG islands were mapped within 500 kb of D7S496. Of these, DRA maps 250 kb telomeric of D7S496 and is expressed in colon but down-regulated in adenomas and adenocarcinomas of the colon (Schweinfest et al. 1993). DRA displays a strong amino acid sequence similarity to two genes encoding sulfate transporters, namely the diastrophic dysplasia sulfate transporter gene (DTDST; Hästbacka et al. 1994) and the rat sulfate anion transporter gene (sat-1; Bissig et al. 1994), and recently, its role in sulfate transport was confirmed (Silberg et al. 1995). The candidacy of DRA as CLD is based on location, expression pattern, and in part, function. It is tempting to speculate that DRA might act as an anion transporter with a significant role in chloride and bicarbonate trafficking in the gut.

A second interesting candidate gene is PRKAR2B, which maps 350 kb centromeric of D7S496 and encodes a regulatory subunit of cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase. Protein kinases are the common denominators of the diverse effects of

cAMP. They are activated by a dissociation of catalytic and regulatory subunits RIIα and RIIβ. Speculatively, PRKAR2B might be involved in the regulation of anion transport, but its location centromeric rather than telomeric of D7S496 makes it a less persuasive candidate. LAMB1 and DLD map ~450 kb telomeric of D7S496, but their known functions (LAMB1 encodes the laminin β1 chain and DLD encodes dihydrolipoamide dehydrogenase) do not immediately suggest a role in chloride diarrhea. Because CpG islands mark the 5′ end of many genes (Bird 1986), the identification of at least six clusters of rare-cutter restriction sites within 500 kb telomeric of D7S496 suggests that many genes remain unrecognized in this region. Potentially, one of them might encode a previously unrecognized product related to CLD.

Positional cloning is increasingly using knowledge of previously cloned genes that become positional candidate genes. Detailed physical maps are necessary tools for identifying such positional candidates. For positional cloning of CLD, we refined a physical map of the critical region and identified known genes as excellent candidates. The YACs in the CLD region proved to have a low rate of chimerism or deletions, and thus the clones will be suitable also for detailed analysis of gene organization. In addition, these clones will enable the isolation of new genes.

## METHODS

### YACs

YACs were obtained from three sources. The "yWSS" YACs were isolated from a collection of chromosome 7-enriched clones (Green et al. 1995) consisting of both human–hamster hybrid cell line-derived YACs and clones isolated from total genomic libraries constructed at Washington University (Green et al. 1994) and CEPH. The "HSC" YACs were from a chromosome 7-specific library (Scherer et al. 1992) constructed at the Hospital for Sick Children (Toronto, Canada). CEPH YACs were from the megaclone collection (Cohen et al. 1993). Contigs are being constructed in each center by systematic approaches using random or end-fragment STSs, polymorphic markers, or gene-specific probes. YAC clones were colony-purified, and DNA was prepared according to standard procedures (Brownstein et al. 1989). The sizes of YACs were determined by pulsed-field gel electrophoresis (Chu et al. 1986).

### Long-range Restriction Mapping

Restriction maps of the YACs were constructed by studying series of partial digestions with the following rare-cutter restriction enzymes: *Not*I, *Eag*I, *Bss*HII, and *Sac*II. Each of these enzymes contains at least two CG dinucleotides in their recognition sequence, making it highly likely that clusters of their recognition sites coincide with CpG islands that tend to associate with the 5′ ends of genes. Electrophoreses were run at 15°C for 20–30 hr at 200 V with 25- to 70-sec pulse time using 1% agarose gels and 0.5 × TBE buffer in a Bio-Rad CHEF–DR2 apparatus. DNA was transferred to nylon membranes (Hybond N+; Amersham) by alkaline blotting. Membranes were hybridized with [α-$^{32}$P]dCTP-labeled vector-specific or locus-specific probes (Table 1) in 1 M NaCl, 1% SDS, and 10% dextran sulfate at 65°C. After high-stringency washes, autoradiographs were exposed for 1–7 days.

### Markers, Genes, PCR Assays, Radiation Hybrids

Primers and hybridization probes for the genes are summarized in Table 1. Insert end fragments of YACs were isolated by ligation-mediated PCR as described (Kere et al. 1992). PCR products were excised from low-melting point agarose gels (NuSieve GTG, FMC) and used directly for labeling and hybridization with Southern filters with *Taq*I-digested YAC DNA. The Stanford radiation hybrid panel G3 was purchased from Research Genetics, and the DNA content of each clone was assayed by PCR for selected markers specific for chromosome 7 (Table 1 ).

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