RESEARCH

Construction of a 750-kb Bacterial Clone Contig and Restriction Map in the Region of Human Chromosome 21 Containing the Progressive Myoclonus Epilepsy Gene

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The gene responsible for progressive myoclonus epilepsy of the Unverricht–Lundborg type (EPM1) is located on human chromosome 21q22.3 in a region defined by recombination breakpoints and linkage disequilibrium. As part of an effort to clone the EPM1 gene on the basis of its chromosomal location, we have constructed a 753-kb bacterial clone contig that encompasses the region containing the gene. Because DNA markers from the region did not identify intact yeast artificial chromosome (YAC) clones after screening several libraries, we built the contig from cosmid clones and used bacterial artificial chromosome (BAC) and bacteriophage P1 clones to fill gaps. In addition to constructing the clone contig, we determined the locations of the *Eco*RI, *Sac*II, *Eag*I, and *Not*I restriction sites in the clones, resulting in a high-resolution restriction map of the region. Most of the contig is represented by a level of redundancy that allows the orders of most restriction sites to be determined, provides multiple data points supporting the clone orders and orientations, and allows a set of clones with a minimum degree of overlap to be chosen for efficient additional analysis. The clone and restriction maps are in excellent agreement with maps generated of the region by other methods. These ordered bacterial clones and the mapping information obtained from them provide valuable reagents for isolating candidate genes for EPM1, as well as for determining the nucleotide sequence of a 750 kb region of the human genome.

Progressive myoclonus epilepsy of the Unverricht–Lundborg type (EPM1) is inherited as an autosomal recessive disorder and is characterized by severe stimulus-sensitive myoclonus, tonic–clonic seizures, and progressive neural degeneration. The age of onset is typically between 6 and 15 years. This condition is one of a group of five different subtypes of progressive myoclonus epilepsy, all of which show progressive neurodegeneration and variable degrees of severity. Unlike the other progressive myoclonus epilepsies, EPM1 is not characterized by biochemical markers such as inclusion bodies or storage material, and diagnosis of the disease is usually based on careful evaluation of the clinical history, typical electroencephalograph abnormalities, and the exclusion of the other four subtypes (Lafora's disease, MERRF syndrome, neuronal ceroid lipofuscinosis, and sialidosis). Identification of the mutated gene responsible for EPM1 will likely contribute to our understanding of the pathophysiology of the disease, give insights into the genesis of seizures in other epilepsies, broaden our knowledge of normal brain function, and aid in the discovery of more effective therapies for different forms of epilepsy.

The locus for EPM1 was assigned to chromo-

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some 21q22.3 by linkage in Finnish families to the polymorphic DNA markers BCEI, D21S154, and D21S112 (Lehesjoki et al. 1991), and its location was refined by further linkage analysis to a 3.5 million base pair (Mb) region defined by the markers MX1 and CD18 (Lehesjoki et al. 1992), with a maximum multipoint lod score of 11.04 at loci D21S154-PFKL. Analysis of an American family with EPM1 defined crossover events that refined the proximal marker to be CBS, shrinking the size of the region containing the gene to approximately 2 Mb (Lehesjoki et al. 1993a). As a result of pronounced founder effects and bottlenecks in the Finnish population history, coupled with population expansion in isolation (de la Chapelle 1993), it can be assumed that most cases of EPM1 arose from a single founder mutation. Results from linkage disequilibrium analysis of 38 Finnish families support this hypothesis and suggest that the EPM1 gene lies within 0.3 cM from the markers D21S25, PFKL, and D21S154 (Lehesjoki et al. 1993b).

Our laboratories are using positional cloning methods to identify the gene responsible for EPM1. Toward this goal, we constructed a bacterial clone contig of 753 kb in the region of chromosome 21 that likely contains the gene. We also determined a high-resolution *Eco*RI restriction enzyme map and the positions of three rare cutter enzyme sites across the entire cloned region.

RESULTS

Strategy

Our laboratories (Zuo et al. 1993; Patil et al. 1994) and others (e.g., see Baxendale et al. 1993; Xie et al. 1993; Murrell et al. 1995) have generated cosmid contigs of several hundred kilobase stretches in a few regions of the human genome by using yeast artificial chromosome (YAC) clones to isolate cosmid clones. However, because we were unable to identify YAC clones in most of the segment of chromosome 21 containing the EPM1 gene, we used an alternative strategy to build a bacterial clone contig of the region. We initially used the available DNA markers from the region as hybridization probes against a chromosome 21-specific cosmid library to isolate cosmids from which to begin building our contig. Cosmids were evaluated by restriction digest with ethidium staining, and those sharing two or more bands of identical size were considered to be overlapping and were arranged into small contig groups. Single cosmid fragments and, in some cases, entire cosmids, were used as hybridization probes to confirm overlaps. Restriction fragments from the ends or near the ends of cosmids were used as hybridization probes in pulsed-field gel electrophoresis (PFGE) mapping experiments to ensure that the cosmids were derived from the appropriate *Not*I restriction fragment, as defined by the map of Ohki and colleagues (Ichikawa et al. 1993). Once confirmation was obtained, these probes were then used to screen the cosmid library to extend the contig. In two regions where we failed to isolate cosmids that would fill in gaps, we obtained DNA sequence information from the cosmids at the ends of each contig, designed sequence-tagged sites (STSs), and screened P1 and bacterial artificial chromosome (BAC) libraries by PCR to obtain clones that completed the contig. The locations of *Eco*RI and several rare-cutter restriction sites were determined by restriction analysis of the entire set of clones.

The Map

These efforts led to the construction of a 753-kb contig with continuous bacterial clone coverage, most of which is represented by multiple overlapping clones (Fig. 1). A total of 108 cosmids, three P1, and two BAC clones comprise the contig. The map contains 14 STSs and a single published hybridization probe, resulting in a map with markers separated by an average distance of 50 kb. Of these, six are new markers that we developed in this study (D21S1988, D21S1953, 2489/90, D21S1978, 2493/4, and D21S1991). Five of the markers described previously are *Not*I linking clones described by Ichikawa et al. (1993; D21S1458/LJ112, D21S1459/LB2T, D21S1460/LJ104, D21S1461/LB85S, and D21S400/LL23SP). Two are described anonymous DNA segments, D21S25 and D21S154, one is the gene PFKL, and one is a CA repeat (D21S1259).

In addition to restriction mapping some cosmids during the construction of the contig, we determined the sizes of the *Eco*RI fragments in all the cosmids, BACs, and P1 clones by digesting them with the enzyme and electrophoresing the digests on long agarose gels. Cosmids known to be overlapping from the screening experiments were loaded on the gels adjacent to one another to simplify the comparisons of the digest patterns. The depth of the contig in clones was great enough that a large fraction of the *Eco*RI fragments were interrupted by at least one cosmid,

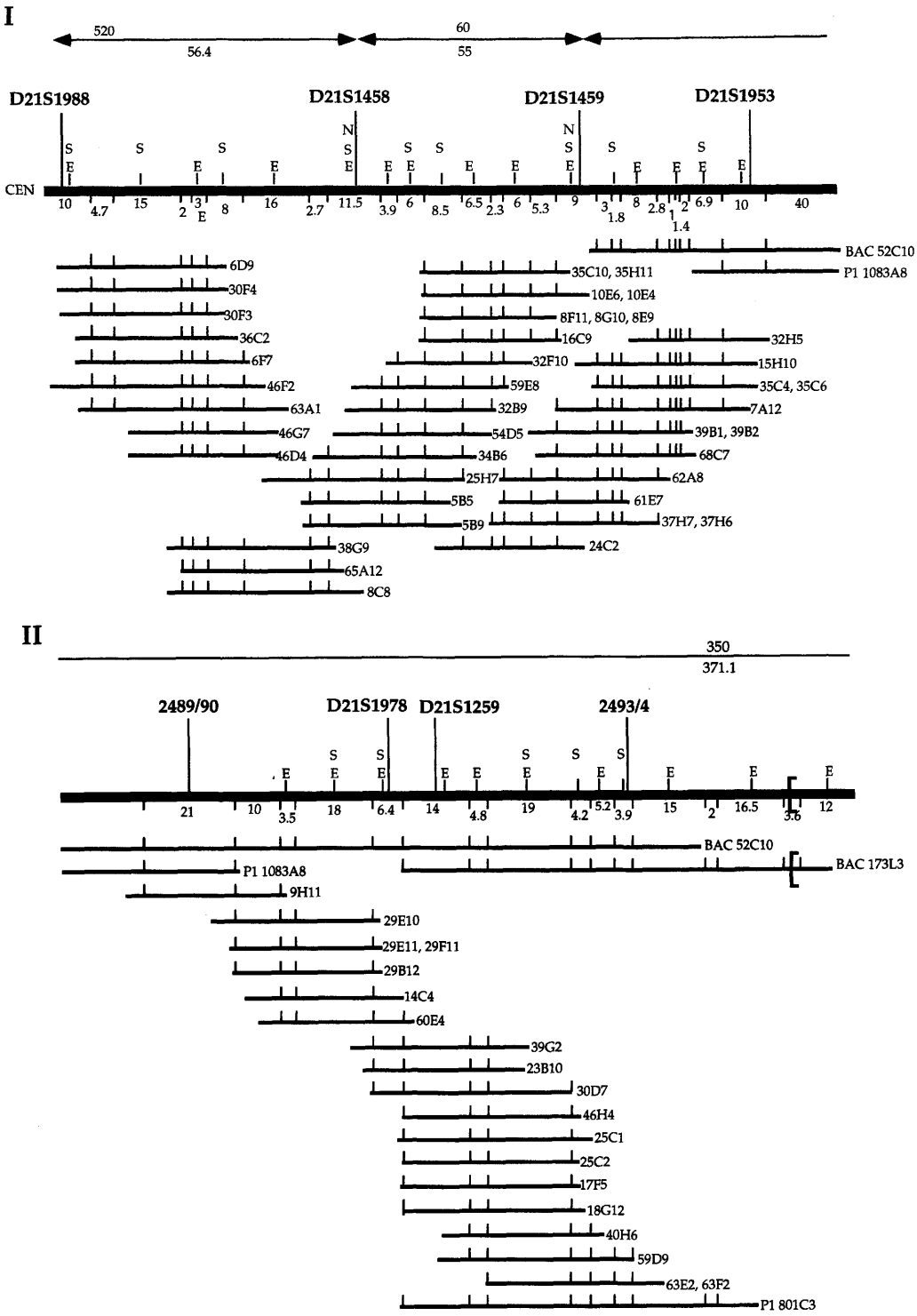
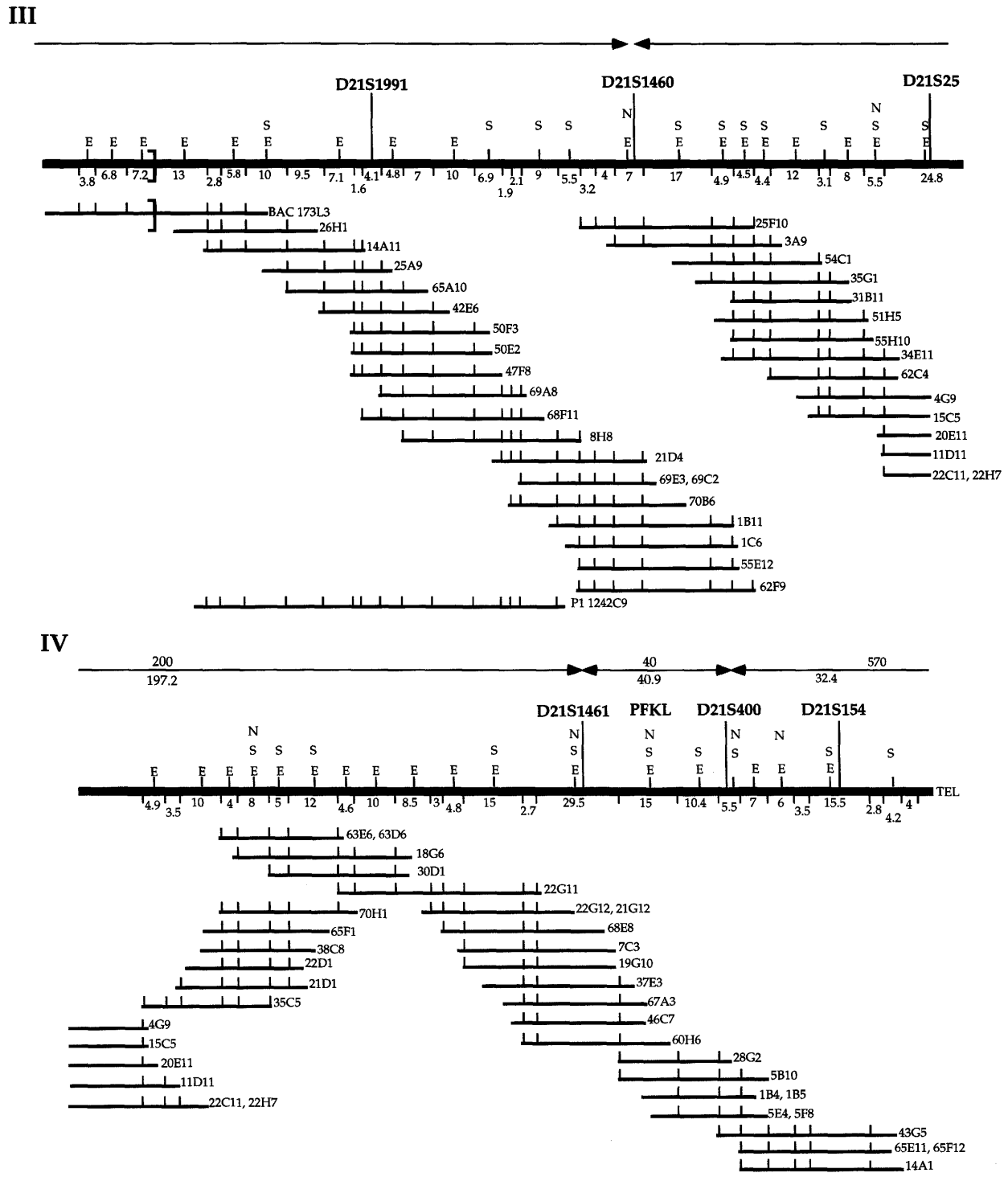


Figure 1 A 753-kb bacterial clone contig of the EPM1 gene region and a portion of the autoimmune polyglandular disease type I gene region on human chromosome 21q22.3. The map extends in the centromeric to telomeric direction from *left* to *right* and is drawn in four contiguous segments labeled I–IV. The top horizontal arrows in each segment designate the *Not*I fragments identified in the map of Ichikawa et al. (1993); the numbers above and below these lines indicate the sizes of the fragments (in kb) determined from their map and our map, respectively. DNA markers are designated by bold letters and numbers, and *Sac*II, *Eag*I, and *Not*I sites are designated by the letters S, E, and N, respectively, above the thick black horizontal line. The *Eco*RI fragments are demarcated by tick marks below the thick line, and their sizes in kb are shown between each pair of tick marks.



Cosmids, BACs, and P1s are shown as horizontal lines with their *Eco*RI sites designated with tick marks and their microtiter well positions from their original library addresses to the *right* of each line. The cases of cosmid clones that have two well position names are likely duplicates of the same clone. All clones with names beginning with a number are cosmid clones, whereas the BAC clone names begin with the letters BAC followed by a well position number, and the P1 clone names begin with the designation P1 followed by a well position number. The orders of the *Eco*RI fragments in the segment of BAC 173L3 that are present in the contig as single clone coverage were not determined and are contained within the brackets.

BAC or P1 end, which allowed the orders of almost all the fragments to be determined. The portion of BAC 173L3 that contains only single coverage contains several *Eco*RI sites whose orders were not determined. This *Eco*RI map provided a framework for identifying the locations of all the rare-cutter sites for *Not*I, *Eag*I, and *Sac*II in the region. Double digests were performed on an overlapping set of cosmids with *Eco*RI and each of the rare-cutter enzymes, and compared to a digest with *Eco*RI alone. In total, we identified 9 *Not*I, 59 *Eag*I, and 35 *Sac*II sites in the 753-kb region.

We determined the total length of the contig to be ~753 kb by adding the sizes of the *Eco*RI fragments. Because the depth of clones was ~10-fold, we were able to use only the internal *Eco*RI fragments of each clone insert to determine distance. This allowed a more accurate estimate of length of the contig to be made than would have been possible if the end fragments of the clones had been used in the calculations. We also calculated lengths for the distances between each of the *Not*I linking clones by adding the sizes of *Eco*RI fragments, allowing a comparison with the *Not*I fragment sizes obtained in the published *Not*I map estimated by PFGE (Ichikawa et al. 1993). The fragment sizes correlated well (Fig. 1, numbers above and below arrows at the top of each map segment). Ichikawa et al. (1993) estimated that linking clone markers D21S1458 and D21S1459 flanked a 60-kb *Not*I fragment, and we calculated the fragment to be ~55 kb. The next distal fragment, flanked by the markers D21S1459 and D21S1460, was reported as 350 kb from the pulsed-field map and compares with 370 kb from the cosmids. The final two fragments, between linking clones D21S1460 and D21S1461 and clones D21S1461 and D21S400, compare at 200 kb versus 197 kb and 40 kb versus 41 kb by the two methods, respectively.

DISCUSSION

We used bacterial clones to obtain a high-resolution clone and restriction map of the region of chromosome 21q that likely contains the gene responsible for EPM1. The argument for the disease gene lying in this contig is based on a combination of standard genetic mapping and linkage disequilibrium data (Lehesjoki et al. 1993a,b). When we began this study, we attempted to isolate YAC clones for the region, and with the exception of a small YAC isolated with a probe for PFKL, we were unable to identify clones containing the other known DNA markers in the region. Similar results have been reported by others (Gardiner et al. 1995). For this reason, we used a combination of STS content mapping and walking with bacterial clone libraries to obtain clone coverage of the region. To avoid walking into inappropriate regions of the chromosome or genome, which can occur especially when hybridization probes are used for walking because of low-copy repetitive sequences, we mapped representative clones from each walk to the predicted *Not*I fragment by PFGE and, in some cases, by radiation hybrid mapping (data not shown). In addition, the agreement in the *Eco*RI restriction maps of the average of 10 cosmid clones covering most of the contig provides an additional measure of confidence that the clones are localized correctly and that the cosmid clones are very likely not rearranged. Furthermore, the presence of identically sized *Eco*RI fragments in the regions of the BACs and P1 clones that overlap with the cosmids suggests that these larger insert clones are also a faithful representation of the regions of the genome from which they are derived.

To build the contig, we screened a total of 6432 clones from the chromosome 21-specific cosmid library, representing an estimated sixfold calculated coverage of the chromosome. However, we obtained ~10-fold redundancy in the clones for most of the region. Thus, for each 50 kb of DNA, there are 10 overlapping cosmids on average, except for the two regions with single coverage by BAC clones. Because we screened a number of cosmids estimated to be about sixfold coverage of the chromosome, our results indicate that this region of the chromosome is more highly represented in the library than are other parts of chromosome 21.

Although most of the contig is represented as multiple overlapping cosmid clones, there are two segments that are not as well represented in our contig. The region containing BAC 52C10 and P1 1083A8 has an ~40-kb segment that is covered only by parts of these two clones, and ~35 kb of the region spanned by BAC 173L3 is covered only by that single clone. We used BAC clones 52C10 and 173L3 as probes to screen the LLNL chromosome 21-specific library in an effort to increase clone coverage in those areas but were unable to identify any new cosmids in these screens. These results suggest that there are portions of this region of chromosome 21 that are difficult to clone in cosmids and is consistent with the notion that complete clone coverage of

the genome for sequencing and other projects will likely rely on the use of libraries constructed with multiple types of cloning vectors.

Ichikawa et al. (1993) identified five *Not*I sites in this region, each of which is associated with the linking clones that we used to begin the cosmid isolation. We identified four additional *Not*I sites not reported by this group, which are those sites that are not digested in genomic DNA from the somatic cell hybrid that Ichikawa et al. (1993) used to construct their *Not*I map. The clustering of these sites on the map helps to identify positions of potential CpG islands, which may be useful for identifying genes in the region.

A cosmid contig for a portion of the region that we presented in this paper, spanning the 400-kb region from D21S460 (LJ104) to D21S154, was published recently (Lafreniere et al. 1995). These investigators employed a cosmid walking strategy similar to the one we used and determined *Hin*dIII and rare-cutter restriction maps of their segment. Because the same cosmid libraries were used in the two studies, it is possible to compare cosmid assignments and orders in the portion present on both maps. We found no inconsistencies in the two maps. In addition to including their region, our contig extends another 350 kb more proximal, toward the centromere in a region that shows high allelic association with EPM1.

This work provides reagents and mapping information that can be used to identify candidate genes for EPM1. In addition, it may also aid in the isolation of the gene for autoimmune polyglandular disease type 1 (APECED; Aaltonen et al. 1994), which maps between D21S49 and D21S171 DNA markers that lie on either side of our contig. Isolation and identification of the EPM1 gene will allow analysis of the protein product, leading to new insights as to the etiology of EPM1 and may provide insights into other epilepsies as well. Identification of the gene and its mutations will also allow definitive diagnosis of this disease. In addition, our work provides a set of minimal overlapping bacterial clones with detailed restriction mapping data that can be used as templates for determining the nucleotide sequence of this region of chromosome 21.

METHODS

Libraries

The arrayed cosmid library LL21NCO"Q" was constructed at the Biomedical Sciences Division, Lawrence Livermore National Laboratory (Livermore, CA 94550), under the auspices of the National Laboratory Gene Library Project sponsored by the U.S. Department of Energy, and was a generous gift of P. de Jong and colleagues (Roswell Memorial Institute, Buffalo, NY). The source of human genomic DNA for this library was obtained from the Wav 17 cell line, a somatic cell hybrid that contains chromosome 21 as the only human component. We screened 6432 clones, which represent about six equivalents of chromosome 21. The P1 library was constructed at DuPont, Inc. (Shepherd et al. 1994); DNA pools from this library were donated by J.-F. Cheng and E. Rubin (Lawrence Berkeley Laboratories, Berkeley, CA) and correspond to 3.5 equivalents of the genome. DNA pools corresponding to about three genome equivalents from the BAC library were purchased from Research Genetics, Inc. (Huntsville, AL).

Hybridizations

The cosmid library was replicated onto Hybond N + (Amersham) membranes, as suggested by the manufacturer, and screened by radiolabeling 30–50 ng of P1 or BAC DNA, or 20–30 ng of cosmid DNA or cosmid restriction fragments, by random priming. Hybridizations were performed in 1 M NaCl, 1% SDS, and 10% dextran sulfate for ~16 hr at 65°C, and repetitive sequences were blocked with excess human placental DNA as described previously (Zuo et al. 1993). Filters were washed three times in 0.1 × SSC, 0.1% SDS, at 65°C for 20 min.

DNA Preparation

Cosmid and P1 DNAs were prepared from 5- and 200-ml cultures, respectively, grown in TB with 50 μg/ml of kanamycin. BAC DNAs were prepared from 100-ml cultures grown in TB with 12.5 μg/ml of chloramphenicol. All DNAs were prepared by alkaline lysis.

Linking clones LJ112, LB2T, LJ104, LL23SP, and LB85S were generously donated by M. Ohki (National Cancer Center Research Institute, Tokyo, Japan). Bacteriophage λ DNA was prepared by standard plate lysis.

PCR Assays

STSs were designed for sequences obtained from cosmid end fragments. Ends of cosmid DNAs (3 μg) were sequenced by using T7 and T3 primers and Sequenase (U.S. Biochemical) as directed by the manufacturer. Conditions for the amplification of the STSs are available from GenBank. The cosmid fragment bearing the STS was confirmed by hybridization of the radioactively labeled PCR product to an *Eco*RI digest of overlapping cosmids.

The primer sequences and PCR conditions for STS 2493/2494 were the following: 2493, 5′-GCTTGTCACTCCCACTCTC; 2494, 5′-CCAGCTTCCAGTTGGTGTC; 30 cycles at 94°C for 30 sec, 58.5°C for 30 sec, and 72°C for 30 sec in 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, and 1% DMSO. The PCR product is ~100 bp. The primer sequences and condtions for STS 2489/2490 were the following: 2489, 5′-GCATCAAGCCATCGGCTTTG; 2490, 5′-TCAATGCAGGGAATACTTATAG, 30 cycles at 94°C for 30

sec, 56°C for 30 sec, and 72°C for 30 sec in 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, and 50 mM ammonium sulfate. The PCR product is ~200 bp.

Construction of Restriction Maps

The *Eco*RI restriction map was built by determining the sizes of the digested fragments in each cosmid, BAC, and P1 on ethidium-stained 15-cm-long 1% agarose gels, 30-cm-long 0.6% agarose gels, and 1% field inversion gels to allow optimal resolution of different sized fragments. The *Eco*RI restriction fragments were ordered by identifying the internal fragments that overlapped in adjacent clones and determining which fragments were localized to the ends of each clone. The end fragments are different in size in each clone because of the fact that *Eco*RI was not used to generate the inserts in the libraries. The high level of coverage in the cosmid clones resulted in at least one clone end localizing to each *Eco*RI fragment throughout almost all of the contig, allowing most of the fragments to be ordered. Although we could order almost all clones and restriction fragments on the basis of these restriction digestion experiments, additional confirmation of overlaps and orders of *Eco*RI fragments was obtained by hybridizing blots of the agarose gels with published DNA markers, cosmid end fragments, internal fragments, and whole cosmids, as well as by determining the locations of *Not*I, *Sac*II, and *Eag*I sites in the cosmids with double digests. In addition, many cosmid fragments were used as hybridization probes to Wav 17 DNA digested with *Not*I and separated on pulsed-field gels.

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