Physical Map of the Human Chromosome 8p12-p21
Encompassing Tumor Suppressor and Werner’s Syndrome Gene Loci

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Abstract

Detailed physical maps of the human genome are important resources for identification and isolation of
genes responsible for diseases and for the study of their structure and function. We constructed a
2.0-Mb high-resolution physical map within the human chromosome 8p12-p21 region extending from marker
D8S131 to D8S283. The map comprises a series of contigs mostly P1/PAC clones, which span the loci of
potential tumor suppressor genes and the Werner’s syndrome gene. Each P1/PAC DNA was defined by
its size, restriction sites, terminal sequences, intermarker distances and location relative to major genes
and markers. The genes on these P1/PAC DNAs were analyzed by an exon amplification method to
determine their locations. The genes newly found by the exon amplification method together with other
known genes, including those of glutathion reductase, a general transcription factor, protein phosphatase
2A β subunit and Werner’s syndrome, were precisely mapped within the contigs. These P1/PAC DNAs
are useful reagents for the generation of new microsatellite markers to narrow the candidate region of the
tumor suppressor gene(s) and/or genes responsible for other diseases, which are believed to exist in this
region by linkage analysis.

Key words: physical map; P1/PAC; exon amplification; 8p12-p21

1. Introduction

Human chromosome 8 contains various genes related to diseases and disorders of medical importance.1 Its short
arm, 8p12-p21, contains the following genes: glutathion reductase (GSR), which is a causative gene for hemolytic
anemia due to glutathione reductase deficiency; clusterin which is involved in atherosclerosis; lutenizing hormone
releasing hormone (LHRH) for hypogonadotropic hypogonadism due to LHRH deficiency; and plasminogen activator
tissue type responsible for plasminogen activator deficiency.1 This region includes also the gene responsible for
Werner’s syndrome (WRN),2,3 a recessive disor-
genes BRCA1 and BRCA2. The BRCA3 gene shows a positive, cumulative, multipoint lod score of 2.51 with two markers of the 8p12-p21 region, suggesting that the BRCA3 gene is in the region of 8p12-p21.

Cohen et al. constructed a contig comprised of CEPH yeast artificial chromosomes (YACs) extending the entire region of human chromosome 8. In general, however, YACs are often found to contain chimeric DNA sequences that make it difficult to construct a complete contig. Consequently, a contig generated in vectors other than YACs is needed to provide additional information on this region of chromosome 8 to accurately locate and order the known genes, as well as to search for new genes. Accordingly, the P1/PAC phage, BAC, or cosmid clones from chromosome specific libraries were chosen to construct a contig map.

In this study, we constructed a 2.0-Mb high-resolution physical map of the human chromosome 8p12-p21 locus made by overlapping P1/PAC DNAs that extend from the stanniocalcin (STC) gene to WRN. We also searched for new genes in this region by an exon amplification method, and then integrated these new genes and known genes, as well as currently available genetic markers, and transcribed the sequences into a physical map.

2. Materials and Methods

2.1. P1/PAC clones and purification

P1/PAC DNA clones were used for the construction of contigs unless otherwise mentioned. The P1/PAC library was screened using a sequence tag site (STS) marker by a polymerase chain reaction (PCR)-based strategy and positive clones were isolated by Genome System, Inc., (St. Louis, MO). The P1/PAC DNAs were isolated by the alkaline lysis procedure of Sternberg with a slight modification, and were further purified by equilibrium centrifugation in CsCl-ethidium bromide gradients for subsequent analysis.

2.2. Characterization of P1/PAC and STSs

DNA sequences of P1/PAC inserts were determined by PCR-based cycle sequencing with T7 and SP6 primers using a Prism Sequencing Kit (Perkin Elmer). The reaction mixture was run and analyzed by an automated DNA sequencer (Model ABI 373, Applied Biosystems, Inc.). The STSs at the ends of P1/PAC were developed from the end fragment sequences determined by the TAIL-PCR method. End-specific STSs were used to screen the P1/PAC libraries.

2.3. Oligonucleotides

Oligonucleotides were purchased from Sawaday Technology Inc. (Tokyo, Japan). The oligonucleotide primers were designed, and the optimal annealing temperature for each set of primers was determined using the OLIGO primer analysis software program (National Biosciences). Table 1 lists the oligonucleotide primer sequences corresponding to the STSs of each P1/PAC end fragment and the amplicon sizes of the PCR reaction. The chromosomal origins of the end sequences and other STSs were assessed by PCR using the genomic DNA from a mouse-human somatic hybrid cell line containing human chromosome 8 described in the following sections.

2.4. Cell lines

The following cell lines were used in our experiments. Mouse A9 (neo 8) cell lines contain a neo-taged chromosome 8 derived from normal human fibroblasts. SCVA2 cell lines were originally derived from SV40-transformed SCID fibroblasts: SC(+8)-2 is a SCVA2 microcell hybrid containing a neo-taged entire human chromosome 8 and SC(R8)-2 is a SCVA2 microcell hybrid containing a neo-taged short arm of human chromosome 8; B16F10(+8) is a melanoma microcell hybrid containing a neo-taged entire human chromosome 8. To maintain a human chromosome tagged with pSV2neo, 800 µg/ml of the antibiotic G418 sulfate (GIBCO, Grand Island, NY) was added to the growth medium. COS-7 cells were used for exon amplification.

All of these cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% heat-inactivated fetal calf serum. All cell lines were cultured in an incubator with 5% CO2 and 95% air. Cells in the logarithmic phase of growth were used to isolate the genomic DNA.

2.5. DNA labeling

The probes for Southern blot hybridization were made either by the random hexamer method or by random primed incorporation of [α-32P]dCTP (3,000 or 6,000 Ci/mmol) with a DNA fragment generated by PCR. The amplified DNA product was purified by electrophoresis in a 3% agarose gel (NuSeive GTG, FMC Corporation) followed by extraction from the gel. Unincorporated 32P was removed by Sephadex-G25 or -G50 spin column chromatography. The specific activity of probes ranged from 108 to 109 cpm/µg DNA. The probes were denatured by boiling for 1 min followed by chilling in ice. They were preannealed at 65°C for 90 min with a 5000-fold excess (w/w) of sonicated salmon sperm DNA before hybridization.

2.6. Pulse-field gel electrophoresis (PFGE)

The P1/PAC DNA clones were purified by CsCl gradient centrifugation, and were characterized by restriction digestion analysis with Not I, Sal I, Sma I, Sac II, and BsaHI. The electrophoresis of P1/PAC DNA digested with these restriction endonucleases was performed on
Table 1. PI/PAC Clones and Specific Oligonucleotide Primer Sequences clone Name; PI; no  mark, PAC;*, BAC;
than 1.2 Mb. The electrophoresis was performed at a circulating temperature of 14 °C using the Bio-Rad CHEFII method using a CHEF DRII system (Bio-Rad, Richmond, CA). All gels were made up in a 0.5 x TBE electrophoresis buffer (89 mM Tris, 89 mM boric acid, 0.2 mM EDTA, pH 8.0). Contour-clamped homologous electric field (CHEF) gel electrophoresis was performed to characterize the Pl/PAC DNAs under the conventional electrophoresis method or 5'-end 32P-labeled oligonucleotides as described by Feinberg and Vogelstein.19

Southern blot hybridization was carried out by standard procedures as described by Sambrook et al.20 Ten micrograms of genomic DNA or DNA 32P-labeled PCR products obtained by the random priming method or 5'-end 32P-labeled oligonucleotides as described by Feinberg and Vogelstein.19

2.7. Preparation of high-molecular-weight DNA and Southern blot analysis

High molecular weight DNA was isolated from the human placenta and human-mouse hybrid cell lines by sarcosyl disruption of the cell membranes followed by three phenol and two phenol/chloroform extractions. Purified DNA was dialyzed against four changes of 10 mM Tris (pH 8.0) and 1 mM EDTA. Southern blot hybridization was carried out by standard procedures as described by Sambrook et al.20 Ten micrograms of genomic DNA or 1 μl of P1/PAC DNA were digested overnight or for 2 h at 37 °, respectively, with a five fold excess of restriction endonuclease. The cleaved DNAs were then separated on 0.7% agarose gels in 1 x TBE buffer. After visualization on an ultraviolet transilluminator, the gels were denatured and neutralized and then were transferred to nylon membranes by capillary blotting. The membranes were hybridized with a radiolabeled DNA fragment as a probe in a solution of 50% formamide, 10 x Denhardt's solution, 0.6 mg/ml sonicated salmon sperm DNA. The filter was washed at high stringency (0.2 x SSC, 0.5% SDS, 65 °C), and was exposed to X-ray film with intensifying screens until the autoradiogram was scanned.

Table 1. Continued.

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2.8. Exon amplification

Exon sequences were amplified by the exon amplification method13,14 from the P1/PAC DNAs that comprised the contig. Individual P1/PAC DNAs were digested to completion with a combination of BamHI and BglII, and the resultant fragments were cloned into pSPL3 (Gibco BRL, Gaithersburg, MD). The DNAs from the transformants were purified using Qiagen columns (Qiagen, Germany) and were used to transfect COS-7 cells by electroporation. The cells were grown for 48 h and the total cytoplasmic RNA was extracted, which was then converted into cDNA with reverse transcriptase. The fragments containing the exons were amplified by PCR with
specific primer pair sets, Next, they were subcloned into the pGEM-T vector and their DNA sequences were determined.

2.9. Somatic cell hybrid mapping

The genomic DNAs from SC(+8)-2 and SC(R8)-2 cells were used for the preparation of template genomic DNA and cDNA for the PCR analysis. PCR was performed in a 10 µl mixture containing 1.5 mM MgCl₂, 20 mM dNTP, 1× of Perkin-Elmer Cetus buffer, 0.3 mM of each primer, 0.25 units of Taq polymerase, and 50-ng of genomic DNA from mouse-human somatic hybrid cells or cDNA as prepared below. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min; and a final elongation at 72 °C for 5 min. Human and mouse genomic DNAs were included as controls. The amplified products were visualized on 1–3% agarose gels. One microgram of mRNA was used to prepare cDNA. Reverse transcription was made by SuperScript™ RT II (BRL) with oligo (dT)₁₂–₁₈ and random hexamers as primers. After the reaction was completed, the mixture was diluted 500 times in distilled water. All the samples were run without reverse transcriptase as a control to check for contamination with genomic DNA. None of these control samples yielded products resulting from genome DNA contamination. Electrophoresis was performed in 3% agarose gels and the DNA was visualized by ethidium bromide-staining.

2.10. Database search of DNA sequence

Sequences obtained from analyses of exons were searched for homology or identity with any known sequences listed in the public databases such as FASTA and BLASTN from the GCG database search program package.²¹

3. Results and Discussion

3.1. The P1/PAC contig map of human chromosome 8p12-p21

The P1/PAC libraries were first screened with primers to obtain P1 DNA clones that contain the D8S339 (=WT251) marker. The marker D8S339 was previously shown to be very near the WRN locus.²² This initial strategy identified three P1 clones: P1-#2587, -#2588, and -#2589. The same P1/PAC libraries were also screened with D8S540 (=GSR2), which is also a marker very close to the WRN locus.²³ Two P1 clones, P1-#3349 and -#3350, containing D8S540 were isolated by this effort. Similar screenings permitted the isolation of P1s containing the other STS markers, including D8S131, D8S1055, GSR1, D8S2144, D8S2138, D8S2156, D8S2168, D8S2174, D8S2150, D8S2162, and D8S283. The terminal sequences of all the P1/PAC DNAs were analyzed by direct sequencing. These P1/PAC DNAs were confirmed to be on chromosome 8 using somatic cell hybrid mapping, and were used to construct a contig. These efforts resulted in construction of a contig (WS contig) consisting of more than 14 P1/PAC DNAs which represent a physical map of WS locus (Fig. 1).

Similarly, another two contigs were made that were on the telomeric side of the above contig: a contig containing the STC gene (STC contig) and a contig containing the D8S131 marker (D8S131 contig). Gaps exist between contigs STC, D8S131 and WRN. We could not complete the contig by covering these gaps because appropriate P1/PAC DNAs could not be isolated from the P1/PAC libraries.

We used two methods to determine the overlap among P1/PAC clones. First, PCR was used to find overlapping P1/PAC clones and to determine the overlap of the clones. We examined the terminal end sequences of P1/PAC clones by the TAIL-PCR method to identify their overlap with each other (Fig. 1 and Table 1). The terminal sequence of each P1/PAC clone provided an STS which permitted us to search for new clones adjacent to the terminal sequence. Repeating these procedures allowed us to group the P1/PAC clones and to extend the contig. Second, we compared the profiles of restriction enzyme digestion of overlapping P1/PAC clones to assess the degree of their overlap: each P1/PAC clone was digested with Not I, BssHII and Kpn I, and the restriction fragment patterns were compared by PFGE (Fig. 2). The overlapping clones typically shared three or more DNA fragments of identical size. In addition, we determined the relative distances between the P1/PAC clones and their order by fluorescence in situ hybridization (FISH) as reported previously by us (Fig. 1).²⁴

All of the STS markers listed in Table 1 and the PCR conditions are available by e-mail from fsugawar@agene.co.jp. Most of the STSs were obtained by sequencing the termini of P1/PAC clones. To use STSs to screen the P1/PAC libraries or to map the STSs, the PCR conditions were optimized to amplify specifically the sequences from human genomic DNA.

3.2. Mapping of newly developed microsatellite markers

The polymorphic CA-repeat markers are important milestones in the map to search for new genes of medical importance. To make the map more versatile, new CA-repeat markers were developed from the P1/PAC clones constituting the contig (Table 2). Identification of such markers was carried out by the method of Hudson and their genotypes were assessed according to Weber and May.⁶ A total of 12 new markers were obtained from 22 P1/PAC DNAs that extend through the 1.0-Mb WRN region, yielding an average of one marker per 100 kb.
Table 3 lists the genes relevant to the exons obtained from P1/PAC DNAs by an exon amplification method in this study. From these results, the exact locations on the contigs of the genes relevant to the exons were determined. Human STC protein is a glycoprotein homologous to the fish STC protein that may be an important regulator of calcium uptake from the aquatic environment. Two exons of human STC gene were obtained from PAC-#5980, which is at about 487 kb of the telomere of the D8S131 marker.12 One exon of the focal adhesion kinase gene (FAK)27 was obtained from PAC-#4974 and PAC-#4976. Eleven exons of the epoxide hydrolase gene (EH)28 were obtained from PAC-#4974 and PAC-#5476. Two exons of apolipoprotein J (ApoJ)29 were obtained from PAC-#5476. These three genes are near to each other and are within 200 kb of the D8S131 marker. We obtained a total of nine exons (AG1-AG5) whose sequences corresponded to those of the expressed sequence tag (EST) of the public databases. The location

Figure 2. High-density physical and transcription maps near the D8S339 and D8S540 STS markers. Detailed profiles of restriction enzyme sites of overlapping P1/PAC clones from TUBBP1 to PPP2CB are shown. The orientation of genes was determined by Southern blot analysis using as probes the PCR products from the 5' and 3' ends of the genes. Human genomic inserts were cloned between Not I and Sal I sites of P1/PAC vector. Each P1/PAC clone was digested with Not I (N), BssHII (B), Kpn I (K), Apa I (A) and Sal I (S) and the restriction fragment patterns were analyzed by PFGE.

These markers were used in the linkage analysis of the Japanese population to narrow the candidate region of the WRN locus. The details of these CA-repeat markers and the genotypic data of Werner's syndrome patients are described by Matsumoto et al.26

3.3. Construction of transcription map by exon amplification

From the physical map obtained, the exact locations of known genes on the 8p12-p21 region were determined. Multiple sets of primers were designed from the sequences of exons obtained by exon amplification from these P1/PAC clones, as well as on the known marker sequences. The nucleotide sequences of these exons were determined, and they were classified into known and novel exons by searching GenBank/EMBL Data Bases. We also confirmed by PCR using as a template genomic DNA or cDNA from mouse somatic hybrid cells containing human chromosome 8 that these exons were, in fact, located on chromosome 8.
Table 2. Distribution of the New Polymorphic Markers in the WRN Region

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Published Markers

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Table 3. The list of exons obtained by amplification from the P1/PAC DNAs of the contigs

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</table>

of ESTs are: F13626 in PAC-#6044; N40395 was in PAC-#6044; T71480, T78553, T86448, T86895 and L44514 were in PAC-#6463/PAC-#6207; and H78562 was in PAC-#6207. Of these, two exons from PAC-#6044 (AG2) were found to belong to one gene, because RT-PCR by primers set up on these exons produced two kinds of DNA fragments containing both of these exons. This gene is reported in the database as a hypothetical protein in the 5' region of RC114 (an early meiotic recombination gene of yeast, database; Z47071). PAC-#6207 contained an exon of the WS-3 gene, which was cloned and characterized by us. The WS-3 protein has an R-G-D (Arg-Gly-Asp) motif in the N-terminal region that seems to confer adhesive properties to macromolecular proteins like fibronectin. Eight exons of a large gene encoding an RNA-binding protein (RBP-MS) were found in PAC-#4386, -#4387, -#2589, -#2587 and -#4497 covering about 200 kb. An exon with a partial sequence of the human beta-tubulin pseudogene (TUBBPI) was found in PAC-#4895 just telomeric to the RBP-MS gene. Nine exons of the GTF2E2 gene were found in PAC-#4370, -#3349 and -#3350, and two exons of GSR were found in PAC-#3350. Three exons of a new gene, Rep-8, were in P1-#4370 and the 10 end of protein phosphatase 2A beta subunit (PPP2CB) was found in P1-#4624 and P1-#4625.
3.4. Discussion and conclusion

We constructed a 2.0-Mb high-resolution physical map of 8p12-p21 extending from the STC gene to WRN. This physical map is mostly consistent with the high-density genetic maps,\(^2,37-39\) a radiation hybrid map,\(^40\) FISH maps,\(^21\) polymorphic STSs\(^41,42\) and EST\(^43\) content maps, which have so far been partially characterized. This construction may help to analyze various interesting genes including tumor suppressor genes, that were reported to be in this region.

Recently, an integrated map composed of YACs including the chromosome 8p12-p21 region was constructed by Imbert et al.\(^44\) According to their map, the GTF2E2 gene was assigned to a location quite different from that assigned by our contig map. This discrepancy in the assignment of the GTF2E2 location may be due to the chimerism and instability of the YAC clones used by Imbert et al. We confirmed that the GTF2E2 gene is located in P1 clone #3349 together with the proximal marker D8S540.\(^23,43\) Our experiment confirmed the stability of P1/PAC clones and their usefulness to construct a contig covering a relatively large genomic area.

The physical map of this study clarified the order of the STS markers: telomere-[D8S131]-[D8S1055]-[D8S339]-[D8S450]-[D8S2144]-[D8S2138]-[D8S2156]-[D8S2168]-[D8S2174]-[D8S2150]-[D8S2162]-[D8S283]-[D8S87]-[ANK1]-centromere; and those of both new and known genes: telomere-[STC]-[FAK]-[EHH]-[APOJ]-[WS-3]-[TUBB]-[RBP-MS]-[GTF2E2]-[GSR]-[Rep-8]-[PPP2CB]-[WRN]-centromere (Figs. 1 and 2, and Table 1).

These results may facilitate the following genetic analyses. First, newly developed polymorphic markers, together with known ones, are expected to contribute to the further narrowing of the tumor suppressor gene(s) candidate region. Second, the P1/PAC DNAs forming the contig map make it possible to obtain exons and to isolate new cDNAs. Indeed, based on this strategy, we have cloned the RBP-MS, Rep-8, and WS-3 genes. Third, the distribution of the unique sites of restriction enzymes, including BssHII, Sac II, Not I and Sal I, suggests the presence of multiple CpG islands,\(^45\) which are often associated with transcribed regions of vertebrate genomes.\(^46\) Evidence suggests that CpG islands may participate in epigenetic mechanisms regulating gene expression.\(^47,48\) Therefore, this information may also help us to locate new transcription units in this region.

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References


