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High-Resolution Physical Map and Transcript Identification of a Prostate Cancer Deletion Interval on 8p22

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A genomic interval of ~1.5 Mb centered at the MSR marker on 8p22 has emerged as a possible site for a tumor suppressor gene, based on high rates of allele loss and the presence of a homozygous deletion found in metastatic prostate cancer. The objective of this study was to prepare a bacterial contig of this interval, integrate the contig with radiation hybrid (RH) databases, and use these resources to identify transcription units that might represent the candidate tumor suppressor genes. Here we present a complete bacterial contig across the interval, which was assembled using 22 published and 17 newly originated STSs. The physical map provides twofold or greater coverage over much of the interval, including 17 BACs, 15 Pls, 2 cosmids, and 1 PAC clone. The position of the selected markers across the interval in relation to the other markers on the larger chromosomal scale was confirmed by RH mapping using the Stanford G3 RH panel. Transcribed units within the deletion region were identified by exon amplification, searching of the Human Transcript Map, placement of unmapped expressed sequence tags (ESTs) from the Radiation Hybrid Database (RHdb), and from other published sources, resulting in the isolation of six unique expressed sequences. The transcript map of the deletion interval now includes two known genes (*MSR* and *N33*) and six novel ESTs.

[The sequences described in this paper have been submitted to the GenBank data library under the accession nos. AF126202–AF126212.]

The short arm of chromosome 8 is a frequent site of allelic deletions in epithelial tumors as diverse as colon (Cunningham et al. 1993; Fujiwara et al. 1993; Chang et al. 1994), breast (Kerangueven et al. 1997; Anbazhagan et al. 1998), prostate (Vocke et al. 1996; Deubler et al. 1997; Prasad et al. 1998), head and neck (Wu et al. 1997; El-Naggar 1998), bladder (Tackle et al. 1996; Wagner et al. 1997), and lung (Ohata et al. 1993). In prostate cancer, loss of heterozygosity (LOH) for markers on 8p is one of the most frequent somatic mutations, occurring in >60% of these tumors (Cunningham et al. 1996). Functional evidence that chromosome 8 contains tumor suppressor gene-associated activity is provided by microcell-mediated chromosome transfer studies, which demonstrate suppression by this chromosome of *in vivo* metastatic ability and *in vitro* invasiveness in the highly metastatic rat prostate cancer cell line AT6.2 (Nihei et al. 1996; Kuramochi et al. 1997), suppression of both tumorigenicity and *in vitro* invasiveness in the COKFu colon carcinoma cell line (Tanaka et al. 1996), reduction of tumorigenicity in the SW620 colon carcinoma cell line, and total suppres-

sion of soft agar clonicity in the HT29 colon carcinoma cell line (Gustafson et al. 1996). Together, these studies strongly suggest that 8p harbors a tumor suppressor gene involved in the genesis of epithelial malignancies.

Subregional deletion analysis of chromosome 8 in a wide spectrum of tumors demonstrates a high degree of LOH for probes within 8p22, including prostate (Bova et al. 1993; Macoska et al. 1995; Cunningham et al. 1996), colon (Cunningham et al. 1993; Fujiwara et al. 1994; Takanishi et al. 1997), lung (Fujiwara et al. 1994; Lerebours et al. 1999; Wistuba et al. 1999), liver (Fujiwara et al. 1994; Pineau et al. 1999), male breast (Chuaqui et al. 1995), breast (Patel et al. 1994; Kerangueven et al. 1997), ovarian (Wright et al. 1998), and urinary bladder (Ohgaki et al. 1999) cancers, although current evidence suggests that there are several discrete, nonoverlapping sites of LOH on chromosomal bands 8pter → p23, 8p22, 8p21, and 8p12 → p11. The general interpretation of these findings is that there is more than one tumor suppressor gene on 8p and additional data are needed to further narrow the critical region(s) to identify the genes (The Third International Workshop on Human Chromosome 8 mapping 1996).

The best evidence to date for a localized tumor

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suppressor gene interval on 8p is provided by Bova and colleagues (1993), who reported a homozygous deletion of macrophage scavenger receptor (MSR) sequences in a case of metastatic prostate cancer. Bookstein and colleagues assembled a YAC contig encompassing 9 cM of genomic sequence (Bookstein et al. 1994), which was used to develop a long-range restriction map of the 2-Mb interval around MSR by pulse-field gel electrophoresis and Southern blotting of YAC DNA (Bova et al. 1996). Genomic probes isolated from this region were then applied to the tumor sample, indicating that the homozygous deletion interval spans 730–970 kb, extending from N877-13 to E20. In addition, supporting evidence is provided by Levy and colleagues (1999), who recently reported another homozygous deletion in the pancreatic cancer cell line MIA PaCa-2, which overlaps with the prostate deletion and encompasses markers D8S549 and D8S1992.

So far only two genes have been located to the interval. One of them, *MSR*, is expressed in macrophages only and does not appear to be a plausible candidate for a tumor suppressor gene in epithelial neoplasm (Emi et al. 1993). Another gene, called *N33*, was isolated in the process of direct cDNA selection of YAC clones encompassing the interval (MacGrogan et al. 1996). *N33* is silenced by aberrant methylation in most colon cancer cell lines and some primary colorectal tumors. However, SSCP analysis and sequencing of the 11 exons of *N33* in 10 metastatic prostate tumor samples did not detect any mutations. Additionally, studies of *N33* replacement in several colorectal lines failed to show evidence of tumor suppressor activity manifested as either in vitro inhibition of cell proliferation or suppression of tumorigenicity in nude mice (MacGrogan and Bookstein 1997). The significance of these findings with regard to oncogenesis remains uncertain.

Efforts are under way to identify additional candidate genes within this interval. Chinen and colleagues reported the results of exon amplification of YAC clones in 8p22, including the N877-13 to E20 region (Chinen et al. 1996). Two interesting candidates emerged from this study, including PDGF-receptor β -like tumor suppressor (*PRLTS*) and centrosome autoantigen *PCM-1* (Ohata et al. 1994; Fujiwara et al. 1995), but both lie outside of the homozygous deletion interval. None of the remaining 43 exons have been evaluated further or mapped precisely.

Here we report the results of our efforts to identify additional candidate genes within the tumor suppressor gene interval. A high-resolution contig of bacterial clones has been prepared that spans the entirety of the deletion interval. The integration of this map with existing radiation hybrid (RH) databases and preparation of a RH breakpoint map facilitated identification of mapped and unmapped ESTs extracted from the Radia-

tion Hybrid Database (RHdb) and provided a physical scale to the map. This physical map was used to localize and identify potential transcript units from published sources, databases, and exon amplification experiments. Our efforts resulted in the assignment of six novel transcription units within the N877-13 to E20 interval, of which five are expressed in epithelial tissues and cell lines and possibly represent novel genes. The complete contig of the deletion interval and the transcript map should prove useful for the further identification and evaluation of candidates for the 8p tumor suppressor gene.

RESULTS

Physical Map

Figure 1A illustrates the homozygous deletion interval that was the target of the physical map effort. Figure 1B shows the names and approximate extent of the selected YAC clones that encompass the deletion interval, of which YAC clones 946_c_9 and 932_e_9 had been used previously to develop a long-range restriction map of the deletion interval depicted in Figure 1C (Bookstein et al. 1994). To prepare a physical contig, PCR was used to screen human genomic BAC (Shizuya et al. 1992) and P1 (Pierce et al. 1992) pooled DNA libraries, as well as a cosmid library prepared from flow-sorted chromosome 8 (Deaven et al. 1986), using the primer pairs listed in Table 1. A total of 39 sequence tagged sites (STSs) were used for library screening: 20 are published, 2 were a gift from Dr. R. Bookstein (unpubl.), 6 were designed based on the published sequences of *MSR* (Emi et al. 1993) and *N33* genes (MacGrogan et al. 1996), and the remaining 11 STSs were developed from the end sequences of BACs, P1, and cosmid clones isolated in this study (Table 1). Bacterial clone end sequences were determined not to contain repetitive elements by BLAST search against nucleotide databases accessible through the National Center for Biotechnology Information (NCBI at <http://www.ncbi.nlm.nih.gov/BLAST/>).

Additional clones were isolated from the BAC and P1 libraries by hybridization-based screening of high-density gridded filters using an *Alu* PCR amplification product of DNA from the YAC clones shown in Figure 1C as a probe. A PAC clone, E1C, included in this contig, was kindly provided by Dr. R. Bookstein (unpubl.). This clone helped to close a gap that was not represented in the P1 and BAC libraries. Finally, hybridization-based screening of an additional BAC library (human, RPCI-11, Research Genetics) allowed isolation of the BAC clone 112I2, which bridged the last remaining gap between telomeric and centromeric portions of the contig between markers 350A20-T7 and 60C12-T7.

The completed physical map of the N877-13 to E20 interval is depicted in Figure 1E as a least-tiling

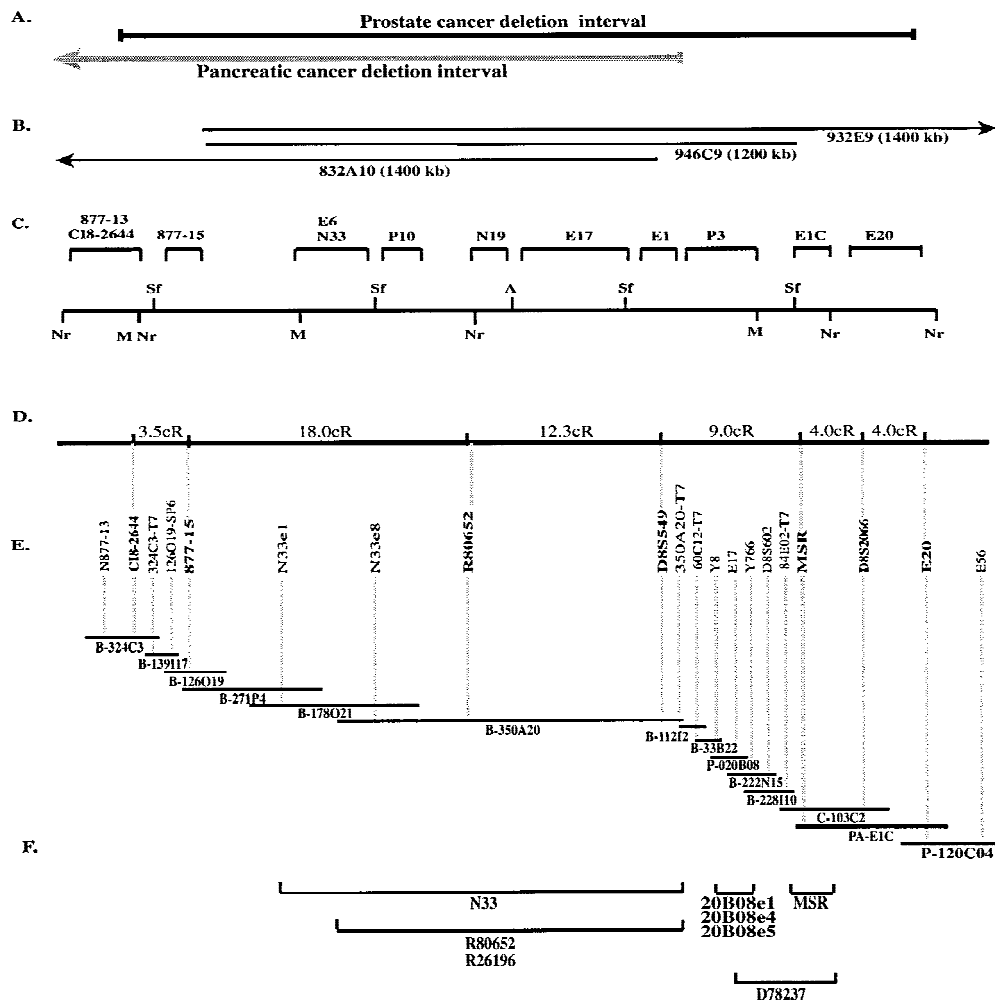


Figure 1 Physical and transcript map of the 8p22 homozygous deletion interval. (A) Extent of the target interval (Bova et al. 1996; Levy et al. 1999). (B) Selected YAC clones that cover the deletion interval and their approximate extent (Bookstein et al. 1994). The numbers refer to the CEPH database YAC name; the approximate sizes of YAC clones are given in parenthesis. (C) Long-range restriction map of the YAC clones encompassing the interval (adapted from Bova et al. 1996). (D) Two-point distances between the indicated markers in cR based on the analysis with the Stanford G3 RH panel. (E) Minimal-tiling path contig of the deletion interval. Only selected markers are shown for simplicity. Those shown in boldface type have also been used for RH analysis. Vertical gray lines connect markers to the bacterial clones with which they tested positive. (Prefix P) PI clones; (PA) PAC clones; (B) BAC clones; (C) cosmid clones. (F) Location of known genes and partial cDNAs described in Tables 3 and 4. Horizontal bars indicate the clones to which transcripts have been localized.

path of selected bacterial clones and markers; a comprehensive map, showing all of the isolated clones and their STS content, is presented in Figure 2. Marker order in Figure 2 was based on STS content of the individual bacterial clones, as well as additional information given in Table 1 (such as derivation from the intronic or exonic sequences of *MSR* and *N33* genes, and from the end sequences of bacterial clones). The contig is about 1.5 Mb in length, and the average marker density across the contig is approximately one per 40 kb of genomic sequence. The complete contig map represents at least twofold coverage of much of the interval by contiguously overlapping BAC, PAC, P1, and cosmid clones.

RH Analysis

RH analysis was performed to define the position of selected markers on the physical map in relation to other markers on the Stanford G3 RH map, and to provide an independent verification of the orientation of the entire contig on the larger chromosomal scale. A total of 15 markers were analyzed, 12 of which were assigned to bins 2, 3, and 4 on the Stanford G3 RH map, including *MSR* (or WI-7228), *D8S549*, and *R80652* markers. Three markers (*E20*, *CI8-2644*, and *N877-15*) were typed on the Stanford G3 RH panel (Stewart et al. 1997) in our laboratory. Note that markers *E20* and *CI8-2644* define the boundaries of the deletion interval. Markers were analyzed using the RH-

Table 1. Complete List of STSs Used for Contig Construction

STS name	Primer		reverse 5' → 3'	Annealing temp. (°C)	Product size (bp)	Source
	forward 5' → 3'					
N877-13	GGACCTAACCCAGATCCAGTA		GCAGCAAAATATCATCTCTC	55	135	Bova et al. (1996)
D8S1731 ^a	CCAAGAAATCATGGAAATC		AGCAAACTATCCCAAGG	55	220 ^b	Stanford G3 RH Map
C18-2644	CCTCTAAGTCCCTGTGTGAG		AAGCGTAATGGGTCTCT	55	186	Bova et al. (1996)
32477	GCTAAGATCACAGAGGAACTGA		ATTGTGTACGATTGCCGTG	55	146	end sequence of BAC 324C3 ^c
126SP6	GCCCTGCTCAGCCATTG		GCAGTCCAGTTTATAGGTCCTG	55	416	end sequence of BAC 126O19 ^c
150E3-17	TTATAGATTGAGGAGTATGGTTGG		TGTATACATAGGTGTATACATAGGT	55	148	end sequence of BAC 150E3 ^c
N877-15	CACGTCTGGCCTTTATTATG		GTAAATGAATGCTTTGAGG	55	288	Bova et al. (1996)
N33e1	GGCCCCCCCCGACACAG		CCCGGGCGCTCTCAGG	65	176	N33, exon 1 sequence ^d
E6	TCCGAGGGCTATATACTTCC		CATCTTGTCTTTGCTCCAAT	55	101	Bookstein et al. (1994)
N33e3	ATGTGGCTGTGCGCAATAAACC		GGCCATAGTAGAACACATCAGT	55	217	N33, exon 3 sequence ^d
D8S1992 ^b	TTTCATCGTCTGAACCTGG		CAGGGTTGAAGCAATCTG	55	300 ^b	MacGrogan et al. (1996)
P10	TTGCAGAGGTTGAGGACAG		CATTAGACATGTCAGAGTC	55	800 ^b	Bova et al. (1996)
N33e8	GCCAGGCTCAGTTTGTG		TGTAGCTTGTAGTTGCCCTTTTC	55	94	N33, exon 8 sequence ^d
115A12-SP6	TGCCCGTAACTTGAATCT		AGCCATCAGCTTGGTCT	55	160	end sequence of P1 115A12 ^c
J28	AGCTTCATCTTCAGAGCAAG		GTACGATAAGGTGACCATC	55	222	gift from R. Bookstein (unpubl.)
N33e11	AGGAACTGGATGAATGGT		TTGCTCGAAATGGATAGT	50	229	N33, exon 11 sequence ^d
D8S549 ^a	AAATGAATCTCTGATTAGCCAAC		TGAGAGCCAACTATTTCTACC	55	170 ^b	Gyapay et al. (1994)
115A12-17	CAGAAACCTTTGTTAACTGTGAG		CTTACCTCATATGTCACACTGT	50	146	end sequence of P1 115A12 ^c
350A20-17	AGTTTTGGGAGCTGTTTTTTGAA		CGCGCCGGCTACTCTC	55	194	end sequence of BAC 350A20 ^c
60C12-17	GAGCTGCTCCGAGAACTTACAT		GTGAGGCTTTACCAACAACAT	55	170 ^b	end sequence of P1 60C12 ^c
N19	GTGAAGGAGGGCCAGTCAAT		CAGGGTTGAAGCAATCTG	55	175	MacGrogan et al. (1996)
60C12-SP6	ACTCTGTTATACACTGATGTAATACAG		TTAGCTGAGACATTTCTGAAG	55	125	end sequence of P1 60C12 ^c
Y8	GCGAAGCTATGTTAAACAT		GGCAAGTAGATGTTACAGAAG	55	150 ^b	gift from R. Bookstein (unpubl.)
E17	CAAGGCATATCAAACTGC		GATAAATGAACTGTCACCTCTG	55	121	Bookstein et al. (1994)
YE766	GACTCTTGCCACCTTGTAAA		ATCTCCAAACCTACTTCTCC	55	89	Bookstein et al. (1994)
E1	TGACACACTTGCATTTGAT		TTCCATTAGTCCCAGTTGTC	55	131	Bookstein et al. (1994)
D8S602 ^a	GACTCTATTTACTATCATTTGACAG		GTATCTTCTGCCCTGGTGAAGAAG	55	300 ^b	Takanishi et al. (1997)
45G06-SP6	GAGGAAATTTAAACAAATGTT		AATTTGCCAGATCTTAG	50	143	end sequence of P1 45G06 ^c
E2d	TGAAGCCATCTGTGGG		GTAAAGAACTGTTAAGTGGAAAG	55	116	Bookstein et al. (1994)
MSR10	GTGGAGCGCCCTCACGAG		TGCACGGCTTGAACACCTGGGTAT	60	139	MSR, exon 10 sequence ^d
84E02-17	CTACCTTGCCACCTTCCAAAACCTA		GAGAACCCAACTGAGGGAACTA	50	154	end sequence of COS 84E02 ^c
MSR5	TTAGAGGAGCGGTTTACAATGT		GAATGTTCCCAATCTTTCAGTCT	55	143	MSR, exon 5 sequence ^d
E3	GCCTGTTTCACTGCAACC		CCTGGCATCTTAACTAGA	55	85	Bookstein et al. (1994)
GATA29A08	CCTGAGTCCAGGAGGTC		CGGAAAACCCCAAGTACA	55	332	Bookstein et al. (1994)
MSR ^a	TTTCATCTATGCAATCC		CAAAATTTCCAGCATGACAACCTG	50	102	Stanford G3 RH Map
D8S2066	TTTTCTCCATCCGGTACTC		CCAACTACGGCATGGTTTTCT	55	170 ^b	Matsumoto et al. (1990)
84E02-13	CCCTGGTGAGACAGCGAGAC		AACTCACTTCCACCCATTTAGG	55	149	Stanford G3 RH Map
E20	TGAATTTGCATAGTCTGCAG		CAGCTTAACAAGGCTCCTA	50	107	end sequence of Cos 84E02 ^c
E56	TTTTGTTGAGACAAATACCC		TGTCACGATGAGGATTGTTA	55	170	Bookstein et al. (1994)

^aDenotes polymorphic markers.^bApproximate PCR product size.^cPrimers derived from end sequences of bacterial clones obtained in this study.^dPrimers designed based on published sequences.

ADDRESS	N877-13	D8S1731	C18-2644	324C3-T7	126O19-SP6	150E3-T7	N877-15	N39e1	E6	N39e3	D8S1992	P10B	N39e8	115A12-SP6	J28	N39e11	D8S549	115A12-T7	350A20-T7	60C12-T7	N19	60C12-SP6	Y8	E17	Y766	E1	D8S602	45G06-SP6	E2D	MSR10	84E02-T7	MSR5	E3	GATA29A08	MSR	D8S2066	84E02-T3	E20	E56			
P-088E03																																										
P-013B01																																										
B-324C3																																										
B-139I17																																										
B-126D19																																										
B-150E3																																										
B-39C5																																										
B-271P4																																										
B-246E11																																										
B-178O21																																										
B-174J12																																										
B-350A20																																										
B-109J20																																										
P-115A12																																										
B-187G23																																										
B-112I2																																										
P-004B01																																										
P-060C12																																										
P-033G07																																										
B-33B22																																										
P-021F04																																										
P-020B08																																										
P-021H04																																										
P-045G06																																										
B-222N15																																										
B-E2D																																										
B-228I10																																										
C-103C2																																										
C-084E2																																										
PA-E1C																																										
P-017E11																																										
P-06A11																																										
P-120C04																																										
P-085H08																																										
P-117F06																																										

Figure 2 Complete list of the isolated bacterial clones and their STS content. Marker names are listed across the *top* row. Markers who order is unresolved in relation to other markers that map to the same clone(s) are indicated by horizontal solid bars at *top*. Clone names are listed in the *left* column in order from telomere (*top*) to centromere (*bottom*). (Prefix P) Pl clones; (PA) PAC clones; (B) BAC clones; (C) cosmid clones. STS content of each clone is indicated by a solid box next to the clone name.

MAP 3.0 statistical package (Lange et al. 1995). Ten markers entered the same linkage group under an lod score criterion of 10.0; two point distances for these markers were calculated with lod scores >7 and are shown in Figure 1D for those located in the deletion interval. The calculated overall distance between markers CI8-2644 and E20 (opposite ends of the deletion interval) is ~ 50.8 cR or 1.47 Mb, using the conversion of 1 cR = 29 kb for the G3 panel (Stewart et al. 1997). Markers entering the same linkage group were analyzed using the RHMAXLIK program. Although it was not possible to order loci with minimum support for the best locus order of $>1.8 \log_{10}$ units, the following marker order was calculated: TEL–D8S511–CI8-2644–N877-15–R80652–D8S549–MSR–D8S2066–E20–SHGC33312–D8S1715–CEN. This level of support reflects the limitations of the G3 panel; however, this order is consistent with the large-scale restriction map of the interval (Bova et al. 1996) and our bacterial contig data.

Placement of ESTs Identified from the Human Transcript Map

ESTs from the publicly available RH consensus transcript map (<http://www.ncbi.nlm.nih.gov/SCIENCE96/>) were integrated into the constructed physical map as follows. The interval to be searched was defined from D8S511 to D8S261, which includes the homozygous deletion interval. The search produced a total of 36 ESTs. BLASTN analysis reduced this number to 27 nonredundant ESTs. All 27 ESTs were then analyzed in more detail using Whitehead-MIT WC8.1 YAC contig assignment data (when available) and placement information from the publicly available RH maps. Based on this information, 19 mapped outside of the deletion interval and, hence, were not pursued further. The remaining eight markers were typed by PCR reactions using DNA from the YAC clones spanning the interval. Three of the eight produced positive hits and identified the following YAC clones: YAC 832_a_10 was positive for marker R80652 and

YAC 946_c_9 was positive for markers stSG4991 and WI-17604. YAC-positive markers were then typed using DNA from the bacterial clones in the interval. Marker R80652 was localized to BAC 350A20 (Fig. 1F), but markers stSG4991 and WI-17604 could not be confirmed by PCR to localize to any bacterial clone in the contig.

Exon Amplification

Bacterial clones from the deletion interval were searched for coding sequences using exon amplification. Five clones covering approximately half of the 1.5-Mb interval were subjected to exon amplification. The resulting 300 clones were digested to release the insert, examined by gel electrophoresis, and subgrouped based on the insert size. A total of 32 clones, each of unique insert size, were sequenced and analyzed. After discarding those containing false splices or vector-derived sequences, five clones remained that contained unique inserts. All five inserts were isolated, labeled, and hybridized to a Southern blot containing *Bam*HI digests of total genomic DNA and DNA from the bacterial clones in the interval (data not shown). Three inserts (20B08e1, 20B08e4, and 20B08e5) appeared to be single-copy genomic sequences, as they recognized single restriction digest bands. Although all three hybridized back to the same clone, P1 20B08 (Fig. 1F), each of them detected a different size band. Two inserts did not hybridize to either genomic DNA or bacterial clones and were not investigated further.

Placement of New ESTs Deposited in RHdb

The RHdb release 13 data set (<http://www.ebi.ac.uk/RHdb/index.html>) was used as a source of unmapped ESTs. The identification of those located either within or near the target interval was done based on the RH breakpoint map using methods similar to those described previously (Horrigan et al. 1999). The analysis produced 23 nonredundant EST markers that were located around the interval and required further confirmation. Two of them were localized independently to the interval by other methods: EST R80652 (RH70072) was obtained from the Human Transcript Map (see above), and EST RH36122 represented a fragment of the *N33* coding sequence. Ten of the resulting 21 markers had genomic assignments to adjacent locations, and 11 were previously unmapped. All 21 markers were tested by PCR on the minimal tiling path of bacterial clones and YAC clones covering the interval. EST (RH36526 or R26196) mapped to YAC 932_e_9 and BAC 350A20 (Fig. 1F). EST RH36122 represented a segment of *DLC-1* (deleted in human liver cancer), a gene reported recently that was suggested as a possible tumor suppressor gene (Yuan et al. 1998). On PCR analysis, this EST did not test positive with either the BAC or the YAC clones and has been reported recently to be

located outside of the interval (P.J. Wilson and G. Chenevix-Trench, unpubl.). The remaining 20 ESTs did not represent any known genes and did not test positive with bacterial clones within the interval. Thus, the analysis resulted in the identification of one novel EST that had not been identified by other methods.

Other Sources of Expressed Sequences

Chinen and colleagues (1996) previously have reported results on the exon-trapping of three YAC clones, of which 931_a_1 and 946_c_9 cover the deletion interval. Among 45 exon-like fragments isolated, 4 represent cDNA fragments of two known genes (*PCM-1* and *PRLTS*) and map outside of the interval. To ascertain which of the remaining 41 fragments lie within the deletion interval, PCR primers were designed for those that had sufficient sequence information (Table 2). A total of 18 primer pairs were successfully prepared, and these were typed on the bacterial contig and YAC clones. Primers designed from exon D78237 identified two overlapping bacterial clones on the contig: BAC 222N15 and BAC 228I10 (Fig. 1F). Two exons (D78242 and D78220) mapped to YAC 932_e_9, but not to any bacterial clone, and are thus thought to be outside of the interval.

Characterization of the Expressed Sequences within the Interval

All ESTs localized to the interval were evaluated for their expression profile by Northern blot analysis. Inserts from the exon amplification-isolated clones 20B08e1, 20B08e4, and 20B08e5 were gel-purified and labeled using random hexamer priming. For the remaining three ESTs, PCR amplification products of genomic DNA with EST primers were used as a probe. PCR products were labeled by random hexamer priming or kinase end-labeling reactions, depending on the size of the product. All probes were hybridized to a multiple tissue Northern blot (Clontech) containing the following tissues: spleen, thymus, prostate, ovary, small intestine, colon, heart, placenta, skeletal muscle, and peripheral blood lymphocytes (PBL). Probes 20B08e1 and 20B08e4 detected an RNA transcript of ~1.2 kb in size, which is expressed in all tissues tested with a high abundance in spleen and PBLs. Probes 20B08e5, D78237, R80652, and R26196 did not detect any message.

EST primers derived from all six sequences (Table 2) were used for RT-PCR analysis using total cellular RNA isolated from the epithelial cancer cell lines SW480 (colon), MDA-MB-231 and ZR-75 (breast), and MIA PaCa-2 (pancreas). Primers designed from sequence 20B08e1 tested negative in all cell lines studied. However, this probe clearly detects a 1.2-kb message on the Northern blot, thus confirming its expression in normal epithelial tissues. The remaining five ESTs

Table 2. PCR Primers Prepared for Evaluation of Expressed Sequences and Their Location to the Physical Map

Sequence name	Primer		Ta ^a (C)	Prod size (bp)	YAC testing ^{b,c}	Contig testing ^{b,c}	Source
	forward 5' → 3'	reverse 5' → 3'					
D78212	TCCAAAACAAAAGACGATGT	GTGGGCAGGTGCCAATAAA	50	121	932 e 9	—	Chinen et al (1996)
D78215	CCACGGCAGGAATGAACAAC	CGCTTAGTCCGCTGAGTCTGT	60	165	—	—	Chinen et al (1996)
D78216	ATCAGTACAAGGGGATCTAACT	ATGTTTTCTTAACGAGGAGACA	50	82	—	—	Chinen et al (1996)
D78221	CCCTCAGGATTGACTAT	TCACGTACACCTTTGGCAT	50	104	—	—	Chinen et al (1996)
D78225	ACAGCCCTCAGGATTGACTA	GAGGCATCATCGAAGGTAAGG	55	88	—	—	Chinen et al (1996)
D78226	AGAGCCCCGAGAGACACCAA	GGCGCTTCCAGGGGAAGA	55	134	—	—	Chinen et al (1996)
D78227	GACCGTTAACACATGTCTATTT	GAAGAGCCAAAGACAGAAACTT	50	143	—	—	Chinen et al (1996)
D78228	TTCAGGTCATTTTCCAGTTT	CTTCCATAGAACGACTTG	50	90	—	—	Chinen et al (1996)
D78229	ATGCGCTACCCCTGAGAAAC	GCAGGTGCTGAATCACAACCTGCA	55	147	—	—	Chinen et al (1996)
D78230	TTTTGAGTAGATTACATAGA	TGGCAAGCGAATTCACCTCT	50	85	—	—	Chinen et al (1996)
D78231	AGAGAAAGGCCACATGAGGACA	AGTGTGGCAGGTCTGATTTCTCC	60	90	—	—	Chinen et al (1996)
D78233	CCTAGACCCTGCCTCAG	CCAAAGGTGGTCAAAGTAGGT	60	81	—	—	Chinen et al (1996)
D78234	TCCTGGCCTAGAGGCTTAA	TGTTTTGTAGGTCTCAATCTTA	50	103	—	—	Chinen et al (1996)
D78237	TATCTGAGCCAACTCCTGCTC	TCITGATAGGGTGGGTAAATA	55	103	932 e 9	B-222N15, B-222110	Chinen et al (1996)
D78239	CTATCCATGCTAAGACATCC	TGATAAAGGAAAGATTGAATG	50	98	—	—	Chinen et al (1996)
D78242	CGCCATCGCAGAGTCA	GTAATTCGCTGCTCCAGTCC	50	111	932 e 9	—	Chinen et al (1996)
D78246	TTCTGCTTTTCTGGACCTGTCC	CACCGGGCTGGGAGGACTGT	55	123	—	—	Chinen et al (1996)
D78247	CCCCAGTTGAGCCATGAGATA	TTGGCAATTTCTAAGTCAGGAA	55	123	—	—	Chinen et al (1996)
R80652	GTCTGATGTCACATCAGATTGAAGG	TGAGCAAAAAGTCTATGCTAAA	55	109	932 e 9	B-350A20	Chinen et al (1996)
WI-17604	TGGGCCAAGACTTTCTG	TAGACTACTTTGGAGATGCAGCC	50	139	932 e 9	B-350A20	Hum. Gene Map, RHdb
20B08e1	CAATGAGAGGGGCTTTGT	GTTTTCCATGGGATGTTCA	50	70	932 e 9	P-20B08	exon amplification
20B08e4	ACTGCCACCACCAGCGTGT	GAGCAAGATGCCAGGGTGGTGTG	55	102	932 e 9	P-20B08	exon amplification
20B08e5	GGGAATTTTAGGTGGTGGTTC	AGGATTCAGGCAATAATGGTTAGC	55	191	932 e 9	P-20B08	exon amplification
R26196	GAATCGTCCATCATAATCCACA	TGACAGCAGGTAAACCGATACC	55	201	932 e 9	B-33B22, P-20B08	RHdb

^a(Ta) Annealing T.^bThe YAC or BAC names indicate that the EST tested positive with this clone.^cNegative signs indicate that the EST did not test positive with any YAC clone or bacterial contig.

showed differential patterns of expression, presented in detail in Table 3. Of particular interest is the fact that ESTs R80652 and R26196 did not show expression in the pancreatic cancer cell line MIA PaCa-2 (Fig. 3). This cell line recently has been reported to contain a homozygous deletion encompassing markers D8S549 and D8S1992 at the telomeric portion of the interval (Levy et al. 1999); therefore, the genomic sequences from which these ESTs are derived are expected to be deleted in this cell line. Representative pictures of the RT-PCR analysis are shown in Figure 3.

Only limited sequence information was originally available for the identified transcript units. Additional sequence information was obtained for exon-like fragments 20B08e1, 20B08e4, R80652, and R26196 by performing 3' and 5' RACE on a Marathon-Ready human fetal brain and placenta cDNA library (Clontech), as is shown in Table 3. The available sequence of all six transcribed units was then used to search the UniGene human sequence collection and The Institute for Genomic Research (TIGR) human gene index database (<http://www.tigr.org/tdb/tdb.html>) to identify all corresponding cDNA clones and detect possible overlap with other clones. The EST R26196 is represented by two cDNA clones, and EST R80652 represented single-clone isolates in the TIGR human gene index database. None of the other sequences (20B08e1, 20B08e4, 20B08e5, and D78237) identified cDNA clones in any of these databases.

Sequences of all six ESTs were used to BLAST search nucleotide and protein databases accessible through NCBI and TIGR. None of the expressed sequences appeared to be identical to any known genes. Exon 20B08e5 showed 95% nucleotide homology to the uncharacterized *Homo sapiens* cDNA clone (GB#AI142134). Some interesting homologies were revealed for the following sequences: 20B08e1 showed 76% homology with the TIGR cDNA clone contig for *H. sapiens* mRNA for phosphatidylinositol-4-phosphate 5-kinase, type II, isoform *c* (TGI#THC154497).

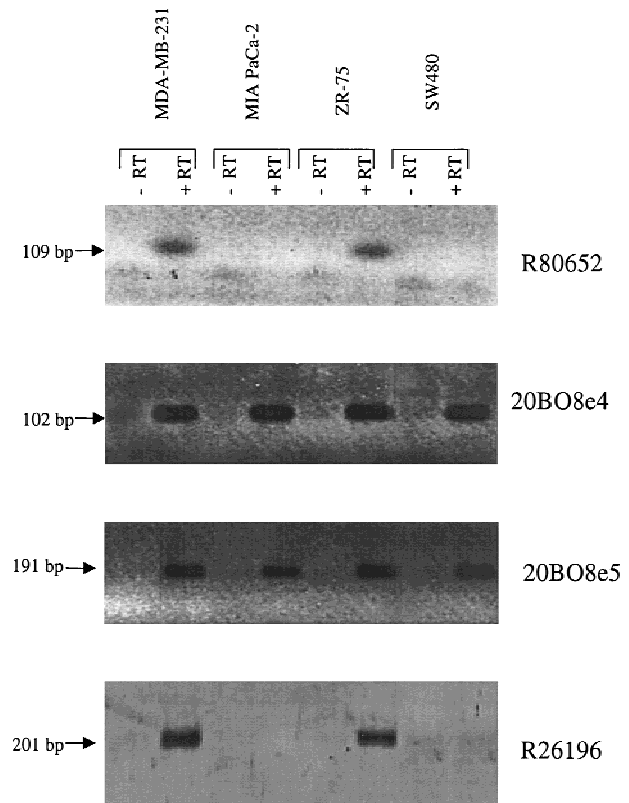


Figure 3 Representative pictures of RT-PCR analysis on selected epithelial cancer cell lines. RT-PCR was performed on total cellular RNA. First-strand cDNA was synthesized using oligo(dT); PCR primers were designed from each partial cDNA clone. The lanes are as indicated. Control reactions without reverse transcriptase and reactions containing water were performed for every sample.

Sequence 20B08e4 did not show any strong homologies at the nucleotide level using the BLASTN program, but the BLASTX search revealed two stretches of 69% and 84% protein homology with *H. sapiens* mRNA for UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl transferase (*GalNAc-T3*) (GB#X92689). Finally, D78237

Table 3. Expression Analysis of Candidate Sequences by Northern Hybridization and RT-PCR

Sequence name	Clone ID ^a	Sequence available (kb)	Northern		RT-PCR			
			band size (kb)	tissue specificity ^b	MB231	PaCa-2	ZR-75	SW480
R80652	146794 (3')	1.86 ^c	none	N.A.	+	-	+	-
20B08e1	—	0.1 ^c	1.2	all tissues tested	-	-	-	-
20B08e4	—	0.5 ^c	1.2	all tissues tested	+	+	+	+
20B08e5	—	0.35 ^c	none	N.A.	+	+	+	+
D78237	—	0.154	none	N.A.	+	-	-	-
R26196	133050 (3')	1.22 ^c	none	N.A.	+	-	+	-

^aIMAGE Consortium clone ID. Clones are available through Research Genetics.

^bTissues used for analysis are: spleen, thymus, prostate, testis, ovary, small intestine, colon, PBL. (N.A.) Not applicable.

^cAvailable sequence information for clones R80652, 20B08e1, 20B08e4, 20B08e5 and R26196 was obtained upon sequencing of the original clones and Rapid Amplification of cDNA Ends (RACE) products.

showed 88% nucleotide homology with sperm surface protein PH-20 mRNA (GB#L13779). BLAST search results for all six ESTs are shown in Table 4.

DISCUSSION

An adequate delineation of a putative tumor suppressor gene site and preparation of a detailed physical map of the interval are the first critical steps in gene identification when using a positional candidate strategy. The 8p22 interval has been thought to contain a tumor suppressor gene, based on recurring LOH in many different types of epithelial neoplasia; however, different reports suggest different locations of the minimal deletion region. A small homozygous deletion at the *MSR* locus, reported in metastatic prostate carcinoma, provides supporting evidence for this site and also sets a logical starting point for the cloning effort. The deletion spans a genomic interval of ~1–1.5 Mb in size from N877-13 to E20 encompassing D8S549 and *MSR*. Two genes previously have been mapped to the interval, but neither one appears to be a tumor suppressor gene.

Here we present the preparation of a high-resolution bacterial contig of the deletion region. The contig provides twofold or greater redundant coverage over much of the interval with contiguously overlapping BAC, PAC, P1, and cosmid clones. The approximate size of this contig is 1–1.5 Mb, and the average marker density across the contig is ~1/40 kb of genomic sequence.

An unresolved contradiction in the literature leaves somewhat questionable the relative position of *MSR* and CI8-2644 in the context of adjacent markers on 8p22 (Fujiwara et al. 1994; Bova et al. 1996), which

may potentially affect the relative placement of some key markers across the deletion interval and orientation of the entire contig on the larger chromosomal scale as well. To obtain independent data addressing this question, we attempted to position CI8-2644, *MSR*, and E20 in relation to each other and to other markers outside of the deletion boundaries, based on the Stanford G3 RH panel. Even though we could not position markers with a high level of support, the obtained order unequivocally agreed with the marker placement on the YAC-based long-range restriction map of the interval (Bova et al. 1996).

The bacterial contig and its integration with the existing EST mapping resources proved to be useful in the identification of the potential coding sequences. A combination of complementary approaches was used in this search, including database analysis, exon amplification of a portion of the interval (primarily excluding the *N33* region), and an extensive search of published literature. ESTs from the D8S511 to D8S261 segment on the Human Transcript Map were identified and confirmed on the bacterial contig. This search produced EST R80652. Other ESTs from RHdb were assessed by means of a RH breakpoint panel, similar to an approach reported recently for a chromosome 5 analysis (Horrigan et al. 1999). This yielded three clones, two of which had been placed on the Human Transcript Map, and one, R26196, which was previously unmapped. Exon amplification provided three sequences, all of which are expressed, based on Northern blot and/or RT-PCR analysis; two of them show homology to known genes. Localization to the interval was confirmed for a fourth exon-like fragment, D78237, which was reported in a study by Chinen and colleagues

Table 4. Results of BLAST Search with Expressed Sequences from the Deletion Interval

Sequence name	Source	BLASTN				BLASTX			
		similarity	identity (%)	length (bp)	P(N)	similarity	identity (%)	length (bp)	P(N)
R80652	Human Transcript Map, RHdb	none				none			
20B08e1	exon trap	none				THC154497; PIP5K	76.2	27	5e-22
20B08e4	exon trap	none				X92689; <i>H. sapiens</i> mRNA for UDP-GalNAc: GalNAc-T3	84	26	2e-8
20B08e5	exon trap	AA278418; <i>H. sapiens</i> cDNA clone IMAGE: 703548	95	71	2e-25	none			
D78237	published	L13779; sperm surface protein PH-20 mRNA	52	88	1e-25				
R26196	RHdb	none				none			

(1996). Interestingly, neither we nor Chinen et al. isolated coding sequences of the *MSR* gene in this approach, even though this gene is present within the interval analyzed.

The transcripts described in Tables 3 and 4 represent six unique sequences localized to the interval, five of which appear to be expressed and therefore derived from cDNAs. Exon-like fragment D78237 did not show expression on either Northern blot or RT-PCR analysis and probably does not originate from coding sequence. None of the identified ESTs are known genes, although three show interesting homologies that will warrant further study.

Sequence 20B08e4 was found to be homologous with *GalNAc-T3*. It is an interesting finding, as GalNAc-transferases initiate mucin-type *O*-glycosylation, which is the formation of an *O*-glycosidic linkage between GalNAc and serine or threonine residues. Aberrant glycosylation and expression of mucins is associated with malignant transformation of epithelial cells from different sites (Hennebicq-Reig et al. 1997; Reis et al. 1998). *O*-glycosylation has also been shown to be important in the folding of proteins such as human chorionic gonadotropin and the binding of cell adhesion molecules such as selectins and selectin ligands (Clausen and Bennett 1996). GalNAc transferases are also required for the synthesis of ganglioside GM2, which is usually significantly elevated in human melanoma cells (Hoon et al. 1992) and is one of the major gangliosides on the cell surface of neuroectodermal tumors (Nakamura et al. 1994).

Sequence 20B08e1 revealed homology with cDNA for *PIP5K*. The phosphatidylinositol pathway is implicated in the regulation of numerous cellular functions and responses to extracellular signals. An important branching point in the pathway is the phosphorylation of phosphatidylinositol 4-phosphate by PIP5K to generate a second messenger, phosphatidylinositol-4,5-bisphosphate (PIP2). PIP5K and PIP2 have been implicated in signal transduction, cytoskeletal regulation, DNA synthesis, and vesicular trafficking. Some findings suggest that a subset of TNF responses may result from the direct association between PIP5K β (isoform of PIP5K) and TNF receptor p55 (Castellino et al. 1997).

Sequence D78237 revealed homology with a sperm membrane protein, *PH-20*. *PH-20* has hyaluronidase activity that is required for sperm penetration through the cumulus cell layer that surrounds the oocyte. This gene was shown to be testis-specific by Lin et al. (1993) and thus is not a likely candidate for a tumor suppressor gene of epithelial origin.

Additional studies are under way to obtain the complete cDNA coding sequence for all of these isolated ESTs and to investigate tumor mutations and biological function, to evaluate them as candidates for the 8p22 tumor suppressor gene.

METHODS

PCR-Based Library Screening

PCR amplification was performed on DNA superpools and plate pools of the BAC (Research Genetics) and P1 libraries (Research Genetics), according to the recommended procedures. Cosmid clones were obtained by PCR screening of 110 plates of the LA08Nco1 flow-sorted chromosome 8-specific cosmid library (Deaven et al. 1986) using a plate-pool strategy. PCR reactions were carried out in a 20- μ l volume consisting of the following: 100 ng of DNA, 1 \times PCR buffer with 1.5 mM MgCl₂ (PE Applied Biosystems), 0.1 mM dNTPs (Pharmacia), 1 μ M of each primer, and 1 unit of AmpliTaq DNA polymerase (PE Applied Biosystems). Amplification was done in a PTC-100 Thermocycler (MJ Research) with an initial denaturation of 94°C for 3 min; then 35 cycles of denaturation at 94°C for 30 sec, annealing for 20 sec, and elongation at 72°C for 30 sec; followed by a 3 min elongation at 72°C. The annealing temperature for each PCR primer pair is listed in Table 1. PCR products were analyzed by electrophoresis on a 2% Nusieve agarose gel (FMC BioProducts) and visualized by ethidium bromide staining.

Hybridization-Based Library Screening

High-density gridded filters were hybridized using an inter-*Alu* repeat amplification product of YAC DNA as a probe. Probes were labeled by random decamer priming using the DECAprime II DNA labeling kit (Ambion). PCR amplification of YAC DNA and hybridization were performed as described by Horrigan and colleagues (Horrigan and Westbrook 1997). Primers used for amplification were previously described (Horrigan and Westbrook 1997). Identified positive clones were ordered from Research Genetics and checked by PCR on cultures for STS content.

DNA Purification

DNA was prepared using tip-500 columns (Qiagen) and the suggested manufacturer's protocol was modified as follows. Glycerol stocks were inoculated in 1 liter of LB media with 25 μ g/ml chloramphenicol (for BAC clones) or 12.5 μ g/ml kanamycin (for P1, PAC, and cosmid clones) and incubated overnight at 37°C. The culture was divided into two 500-ml preparations, precipitated, resuspended in P1 buffer containing 1 mg/ml lysozyme, and incubated at 37°C for 15 min. DNA was eluted from the columns by adding three 5-ml aliquots of QF buffer preheated to 65°C.

Generation of BAC, P1, and Cosmid End Sequences

P1 and cosmid clones were sequenced manually, using SequiTherm Cycle Sequencing kit (Epicentre Technologies). Five fmoles of template and 1.5 pmoles of kinase-labeled primer were used for each sequencing reaction; samples were prepared as described in the manufacturer's protocol. The following primers were used: P1 SP6 (5'-GGCCGTCGACATTAGGTGACAC-3') and P1 T7 (5'-CCGCTAATCGACTCATATAGGG-3') primers for P1 clones and P1 T7 and T3 (5'-ATTAACCCTCACTAAAGGGA-3') primers for cosmid clones. PCR reactions were carried out in the Perkin-Elmer 480 thermocycler at the following cycle conditions: initial denaturation at 95°C for 3 min; 20 cycles of 95°C for 30 sec, 50°C for 30 sec, 70°C for 1 min; 10 cycles of 95°C for 30 sec, 70°C for 1 min. After the PCR reaction, loading dye was added to the samples and they were separated on a gel or stored at -20°C.

Samples were analyzed on a 6% Long Ranger gel (FMC Bio-Products) with 8 M urea at 80 V for 1 hr and 20 min. Dried gels were exposed to Kodak X-Omat film for 12 hr at -20°C with an intensifying screen.

BAC end sequencing was performed on the automated ABI 373 DNA sequencer. The ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) was used. Three micrograms of BAC DNA and 50 pmoles of primer were used in a total volume of 40 μl . The following primers were used: T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') (Boysen et al. 1997). PCR reactions were carried out in the PTC-100 thermocycler (MJ Research) under the following cycle conditions: initial denaturation at 94°C for 4 min; 25 cycles of 96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min. After cycling, PCR reactions were purified by ethanol precipitation (protocol 1) as described in the manual.

Southern Hybridization

Genomic or bacterial DNA was digested with either *Bam*HI or *Hind*III in the presence of spermidine. Restriction fragments were then fractionated through a 0.7% agarose gel and transferred to a Hybond N+ nylon membrane (Amersham) under alkaline conditions. Probes were labeled by random decamer priming using the DECAprime II DNA labeling kit (Ambion). Hybridization and washing were performed at 65°C as described in Hybridization-Based Library Screening (above), without the prehybridization step.

Northern Analysis

Human MTN (multiple tissue Northern) blots (Human II 7759-1 and Human 7760-1, Clontech) were hybridized in ExpressHyb hybridization solution (Clontech) according to the manufacturer's protocol.

RT-PCR

Total cellular RNA was isolated using Trizol reagent (BRL, Life Technologies) following the suggested protocol. First-strand cDNA synthesis was performed using SuperScript Preamplification System and oligo(dT) primer (BRL, Life Technologies) according to the supplied protocol. First-strand cDNA was amplified in the standard PCR reaction using 10% of the first-strand reaction and AmpliTaq DNA polymerase (PE Applied Biosystems). The sequences of primers used and annealing temperatures are indicated in Table 2. Control reactions without reverse transcriptase and reactions containing water instead of cDNA were performed for every sample. RT-PCR products were analyzed by electrophoresis on a 2% Nusieve agarose gel (FMC BioProducts) and visualized by ethidium bromide staining.

Exon Amplification

Exon amplification was performed using GIBCO BRL Exon Trapping System (BRL, Life Technologies) according to the manufacturer's and published protocols (Buckler et al. 1991; Church et al. 1994). Briefly, DNA from bacterial clones was restricted using a *Bam*HI-*Bgl*II double digest. The resulting fragments were subcloned into the vector pSPL3. Individual transformants were pooled, and episomal DNA extracted from these pools was electroporated into COS-7 cells. Cytoplasmic RNA was extracted and used as a template for reverse

transcription followed by PCR using vector-specific primers. Primary PCR amplification was *Bst*XI-treated to reduce the recovery of vector and cryptic splicing products. The product of the secondary PCR amplification with nested primers was cloned into the pAMP10 vector. Individual colonies were transferred to 96-well plates, propagated, and stored as 20% glycerol stocks at -20°C . To minimize the number of false positives, colony filters were hybridized with kinase-labeled PCR product derived from the amplified HIV-intron portion of the vector pSPL3 with primers SD6 (5'-TCTGAGTCACCTG-GACAACC-3') and SA2 (5'-ATCTCAGTGGTATTTGTGAGC-3'). To size-select material for sequencing, 96-well plates with single clones were PCR amplified using primers designed from the cloning site flanking sequences of the vector pAMP10: EXO6 (5'-GCCAGTGAATTGAATTTAGGTG-3') and EXO7 (5'-CAAGCTCTAATACGACTCAC-3'). PCR samples were run on a gel and inserts were grouped based on size. Hybridization-negative clones from different insert-size groups were chosen for sequencing. Clones were sequenced on both strands using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) following the manufacturer's instructions. Sequences were analyzed using BLAST programs (Altschul et al. 1990).

RACE analyses (5' and 3') were performed using Marathon-Ready cDNA (Clontech) and Advantage KlenTaq Polymerase Mix (Clontech) according to the manufacturer's instructions. First-amplification primers and nested primers were designed from the exon amplified and sequenced clones. The following primers were used for 20B08e1: sense ex1-RACE F (ATGGGATGTTTCATGAGAAAGATTT), annealing temperature = 58°C , and antisense ex1-5' RACE (CTGCCAGTGAGAGGGGCTTTG), annealing temperature = 62°C ; nested sense nex1-3' RACE (ATGGACAGGAGACTGCAAA-CAAA), annealing temperature = 60°C ; and nested antisense ex1-RACE R (ATGGGATGTTTCATGAGAAAGATTT), annealing temperature = 62°C . For 20B08e4: sense ex4 F (ACTGGCCAC-CACCAGCGTGAT), annealing temperature = 65°C ; antisense ex4-5' RACE (GCTGGCATCATCCACCAGTATGATCT), annealing temperature = 65°C ; nested sense nex4-3' RACE (GTGTTCCACAACGAAGCCTGGTC), annealing temperature = 63°C ; nested antisense ex4R (GAGCAAGATG-GCAGGGGTGGTGTG), annealing temperature = 68°C .

RH Analysis

PCR score data for the unmapped markers were obtained in our laboratory upon amplification of the Stanford G3 radiation hybrid panel (Research Genetics). The G3 panel was amplified at least twice for each marker. PCR amplification was carried out in 20 μl reactions under standard conditions. The ability of each marker to amplify DNA from an individual hybrid was scored as 0 (no amplification), 1 (clear amplification), or 2 (uncertain). PCR scores for the mapped markers were extracted from the RHdb depository. All PCR scores were analyzed using the RHMAP statistical package (Lange et al. 1995). The RH2PT program was used to calculate two-point distances and linkage groups. Size estimates between markers were calculated based on the conversion of 1 cR_{3000} = 270 kb for the Genebridge panel (<http://www-genome.wi.mit.edu>) and 1 cR_{10000} = 29 kb for the Stanford G3 panel (Stewart et al. 1997). The RHMAXLIK program was used to compute marker order with a maximum likelihood. The equal retention model and the stepwise ordering option were applied; the following conditions were chosen: max \log_{10} likelihood difference to

save order = 5; max log₁₀ likelihood difference to print order = 3; max log₁₀ likelihood support to add locus = 1.

Identification of Unmapped ESTs from RHdb

The identification of unmapped ESTs located around the target interval was done in a manner similar to that described previously (Horrigan et al. 1999). In brief, a segment on the Whitehead-MIT Genebridge4 RH map was defined, which included all positioned markers across the 8p22 chromosomal band, including MSR and D8S549. The RH mapping data were analyzed for all 24 markers in this segment, and the panel of 13 radiation hybrids with breaks within the defined interval was assembled, thus representing the breakpoint panel, which was used later at the last step of analysis. Next, 12 radiation hybrids were identified that had positive scores with all 24 core markers and presumably contained the interval in its entirety. Then, the GB4 subset of the chromosome 8-linked markers (total of 2209 markers) was extracted and all markers positive for all 12 8p22-containing hybrids were identified. These 241 markers were then analyzed using the RH2PT program to identify those within 10 cR (~3 Mb) distance of MSR and/or D8S549, with lod scores >12. A total of 32 markers were so identified and their PCR scores were compared to the breakpoint panel. Six markers were ruled to be located outside the interval based on the fact that their PCR scores did not match PCR scores of MSR and D8S549. The remaining 26 markers had identical PCR scores and were equally likely to be close to the deletion interval, and their position could not be resolved any further, given the low resolution of the Genebridge4 RH panel. Analysis revealed 3 redundant entries; the remaining 23 markers were all ESTs and required further confirmation by PCR typing on the bacterial contig.

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