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Cloning and Gene Mapping of the Chromosome 13q14 Region Deleted in Chronic Lymphocytic Leukemia

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Frequent deletions and loss of heterozygosity in a segment of chromosome 13 (13q14) in cases of B-cell chronic lymphocytic leukemia (CLL) have suggested that this malignancy is caused by inactivation of an unknown tumor suppressor gene located in this region. Toward the identification of the putative CLL tumor suppressor, we have constructed a high-resolution physical map of YAC, PAC, and cosmid contigs covering 600 kb of the 13q14 genomic region. In addition to densely positioned genetic markers and STSs, this map was further annotated by localization of 32 transcribed sequences (ESTs) using a combination of exon trapping, direct cDNA selection, sample sequencing of cosmids and PACs, and homology searches. On the basis of these mapping data, allelic loss analyses at 13q14 using CLL tumor samples allowed narrowing of the genomic segment encompassing the putative CLL gene to <300 kb. Twenty-three ESTs located within this minimally deleted region are candidate exons for the CLL-associated tumor suppressor gene.

INTRODUCTION

B-cell chronic lymphocytic leukemia (CLL), which is the most frequent type of leukemia in adults of the western world, results from the clonal expansion of a subset of B-cells carrying the CD5 cell-surface marker (Foon *et al.*, 1990; Dighiero *et al.*, 1991). In its early phase, CLL is characterized by an indolent clinical course lasting several years, and then the disease becomes more aggressive and is often fatal.

The pathogenesis of CLL remains unknown, and no specific genetic alterations involving proto-oncogenes or tumor suppressor genes have been identified. Earlier studies suggested an association between CLL and mutations of the BCL-1 and BCL-2 oncogenes, but either these alterations were later shown to be rare or the cases were misdiagnosed (Gaidano *et al.*, 1994). CLL is sometimes associated with inactivation of the tumor suppressor gene p53, but such lesions are likely to represent secondary events during tumor progression, because they are observed in rare cases, most of which represent late stages of the disease (Gaidano *et al.*, 1991). Trisomy of chromosome 12, which is commonly detected in cytogenetic analyses of large CLL panels (Juliusson and Gahrton, 1993) and is associated with cases displaying atypical lymphocyte morphology, also appears to be related to tumor progression (Jabbar *et al.*, 1995; Matutes *et al.*, 1996; Garcia-Marco *et al.*, 1996).

The abnormality most frequently detected cytogenetically in CLL (25% of the cases) is deletion of a portion of band 13q14 (Juliusson *et al.*, 1990). Moreover, the results of PCR analyses showed allelic loss of genomic markers within 13q14 in a significant number of additional cases (Jabbar *et al.*, 1995; Liu *et al.*, 1995; Devilder *et al.*, 1995; Bullrich *et al.*, 1996). These observations suggested that a tumor suppressor gene involved in leukemogenesis is present in 13q14, which is different from the colocalized retinoblastoma susceptibility gene RB1. RB1 has been eliminated as a CLL candidate gene, because the commonly deleted region in CLL has beeen mapped to a 1-Mb interval that includes the loci D13S272 and D13S25 (Juliusson *et al.*, 1990; Brown *et al.*, 1993; Chapman *et al.*, 1994; Liu *et*

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al., 1995; Devilder *et al.*, 1995; Bullrich *et al.*, 1996), which are telomeric to RB1.

To define the 13q14 region commonly deleted in CLL more precisely, and to generate reagents for positional cloning of the CLL-associated gene, we have constructed a YAC, PAC, and cosmid contig map covering the entire candidate region. This map was used to determine the order and relative positions of established STSs and ESTs within the region and to develop markers for narrowing the region that is minimally deleted in CLL. Using these probes, we screened a large panel of CLL samples and restricted the genomic segment encompassing the putative CLL tumor supressor gene to <300 kb. This gene could be included among the several novel transcribed sequences that we have identified within this region.

MATERIALS AND METHODS

Chromosome 13 DNA clones. YAC clones were selected from the CEPH I human YAC library, the CEPH human mega-YAC library (Albertsen *et al.*, 1990), and the ICRF human YAC library (Larin *et al.*, 1991). Segments RPCI1 and RPCI3 of the arrayed human genomic PAC library in vector pCYPAC2, a derivative of pCYPAC1 (Ioannou *et al.*, 1994), were made available by Dr. P. deJong (Roswell Park Cancer Institute, Buffalo, NY). A chromosome 13-specific cosmid library in vector sCos-1, LA13NC01, which was prepared from flow-sorted chromosome 13 DNA of the hamster–human hybrid cell line HHW686, was provided by Dr. L. Deaven (Human Genome Center, Los Alamos National Laboratory, NM).

DNA preparation. Cosmid and PAC DNA was purified on Qiagen Tip 20 or 100 columns according to the manufacturer's instructions, except that for isolation of high-molecular-weight PAC DNA the volume of buffers P1, P2, and P3 was doubled and the pH of elution buffer QF was adjusted to 9.2. To determine insert sizes, cosmid and PAC DNA was digested with *Not*I and analyzed using the FIGE Mapper Electrophoresis system (Bio-Rad, Hercules, CA).

Map assembly. Details of the procedures for library screening, clone characterization and mapping, and use of software for map assembly have been described previously (Fischer *et al.*, 1994, 1996). CEPH YACs previously mapped to 13q14 were identified in public databases of the Columbia University Genome Center (http://genome1.ccc.columbia.edu/~genome/) and the Center for Genome Research at the Whitehead Institute for Biomedical Research (http://www-genome.wi.mit.edu/). Additional YAC clones were identified in the ICRF library by screening PCR pools, and their correspondence to the region of interest was validated by PCR with primers specific for the loci D13S1168, D13S1150, and D13S272. To select a comprehensive set of PAC and cosmid clones underlying these YACs, chromosome 13 cosmid and genomic PAC libraries were hybridized with inter-*Alu* PCR probes from the CEPH YACs 6g10, 752e1, 775c8, 922a8, and 859d12; randomly labeled DNA probes from ICRF YAC 66a8 and CEPH YAC 745e3 purified by PFGE; and probes for STSs across the region (shown in layer 1 of Fig. 1). The authenticity of hybridizing cosmids and PACs was further validated by PCR analyses with STS primers. The selected cosmids and PACs were gridded at high density on minifilters and assembled into contigs aligned to the YACs by riboprobe matrix hybridization (Fischer *et al.*, 1994), using the integrated mapping program IMP (Zhang *et al.*, 1994) to analyze the data. Gaps between cosmid contigs were filled by using riboprobes representing terminal regions of cosmid clones to screen further the chromosome 13 cosmid library.

Tumor samples. The diagnosis of CLL was made according to the criteria of the Fifth International Workshop on Chronic Lymphocytic Leukemia and the National Cancer Institute-sponsored Working Group (Montserrat *et al.*, 1992; Cheson *et al.*, 1996). Peripheral blood samples from 156 CLL patients were fractionated by density gradient centrifugation on Ficoll–Hypaque (Pharmacia, Piscataway, NJ). The mononuclear cell layer was analyzed by flow-immunocytometry to identify the percentage of neoplastic lymphocytes. Among individual tumor samples, the fraction of CD19/CD5-positive cells ranged from 48 to 98%, with a median of 83%. Normal granulocytes in the postgradient pellet were purified by osmotic lysis of red blood cells. Cellular DNA was extracted by cell lysis, proteinase K digestion, "salting out" extraction, and ethanol precipitation as described (Miller *et al.*, 1988).

Allelic loss analysis. Allelic loss in 13q14 was assayed by PCR analysis of tumor cell DNA and normal DNA from the same patient with polymorphic markers corresponding to loci D13S273, D13S272, D13S319, AFM301WB5, and D13S294. The PCR primer sequences were obtained from the Genome Database (http://gdbwww.gdb.org/), the Whitehead Institute, and the CEPH–Généthon integrated map (http://www.cephb.fr:80/). The PCR conditions and the analysis of amplification products were as described (Gamberi *et al.*, 1997), except that 25 PCR cycles were performed using annealing temperatures of 60–64°C. Deletions in the tumor samples were detected from the absence or marked reduction in intensity of one or two allelic PCR fragments, compared with the sample of normal DNA from the same individual. Allelic dosage was also measured by comparative densitometric analysis of Southern blots of *Eco*RI-digested tumor DNA and normal DNA from a lymphoblastoid cell line. The blots were hybridized individually with 12 cosmid-derived probes, and the signal intensity was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized to the signal of a reference probe derived from a 1.3-kb *Sac*I/*Stu*I fragment isolated from the *Sac*I4.0 probe of the BCL-6 gene on chromosome 3q27 (Ye *et al.*, 1993), which shows no alterations in CLL. Standard deviations calculated from these measurements were <20%. Thus, a 40–60% reduction of signal intensity in tumor versus normal DNA was considered a monoallelic deletion, while >60% reductions were scored as biallelic deletions (residual signal from normal cells contaminating the tumor samples was detectable). A probe for D13S1150 was generated by PCR amplification using specific primers listed in databases (see above), while probes 175B12/1, 140F11-T3, and 173a12-82 were generated with primers shown in Table 1. Probe pH2-42 is a 2.2-kb *Hin*dIII fragment that corresponds to locus D13S25 (Lalande *et al.*, 1986).

DNA sequencing. Cosmid insert ends and cDNA clones were sequenced using T3 and T7 primers with FS dye terminator kits on Model 377 DNA sequenators (Applied Biosystems, Inc., Foster City, CA). To determine the entire sequence of cosmid inserts after shotgun cloning into M13mp19 (Deininger *et al.*, 1983), cosmid DNA was first sonicated and then 1- to 4-kb fragments were selected by gel purification. Sequences were edited and assembled with the phred and phrap software packages (developed by P. Green and B. Ewing, Washington University, St. Louis, MI). Vector sequences were screened out using the Cross-Match program. Homology searches were performed using the NCBI Website interface to the BLAST (Altschul *et al.*, 1990) server.

cDNA selection and exon trapping. Purified DNAs of YAC 66a8, PAC p208p23, and a mixture of PACs p157m7 and p246m14 (see Fig. 1) immobilized on nylon filters were used for direct cDNA selection from the human fetal liver–spleen library NFLS (Bonaldo *et al.*, 1996) as described (Bonaldo *et al.*, 1994). Exon trapping was performed using cosmid DNA and the Exon Trapping System kit (Life Technologies, Gaithersburg, MD) according to the manufacturer's specifications.

Characterization of expressed sequences. Segments of genes residing in the region of interest were identified by searching the EST database (dbEST) with sequences obtained from cosmids, by direct cDNA selection, and by exon trapping. Expressed sequences (shown in layer 2 of Fig. 1) were amplified by PCR using primers listed either in Table 1 or in public databases, and the products were used as probes for backmapping by Southern analysis of *Eco*RI digests of cosmid DNAs and for Northern analysis, to examine expression profiles. For Northern blotting, total and poly(A)$^+$ RNAs were isolated

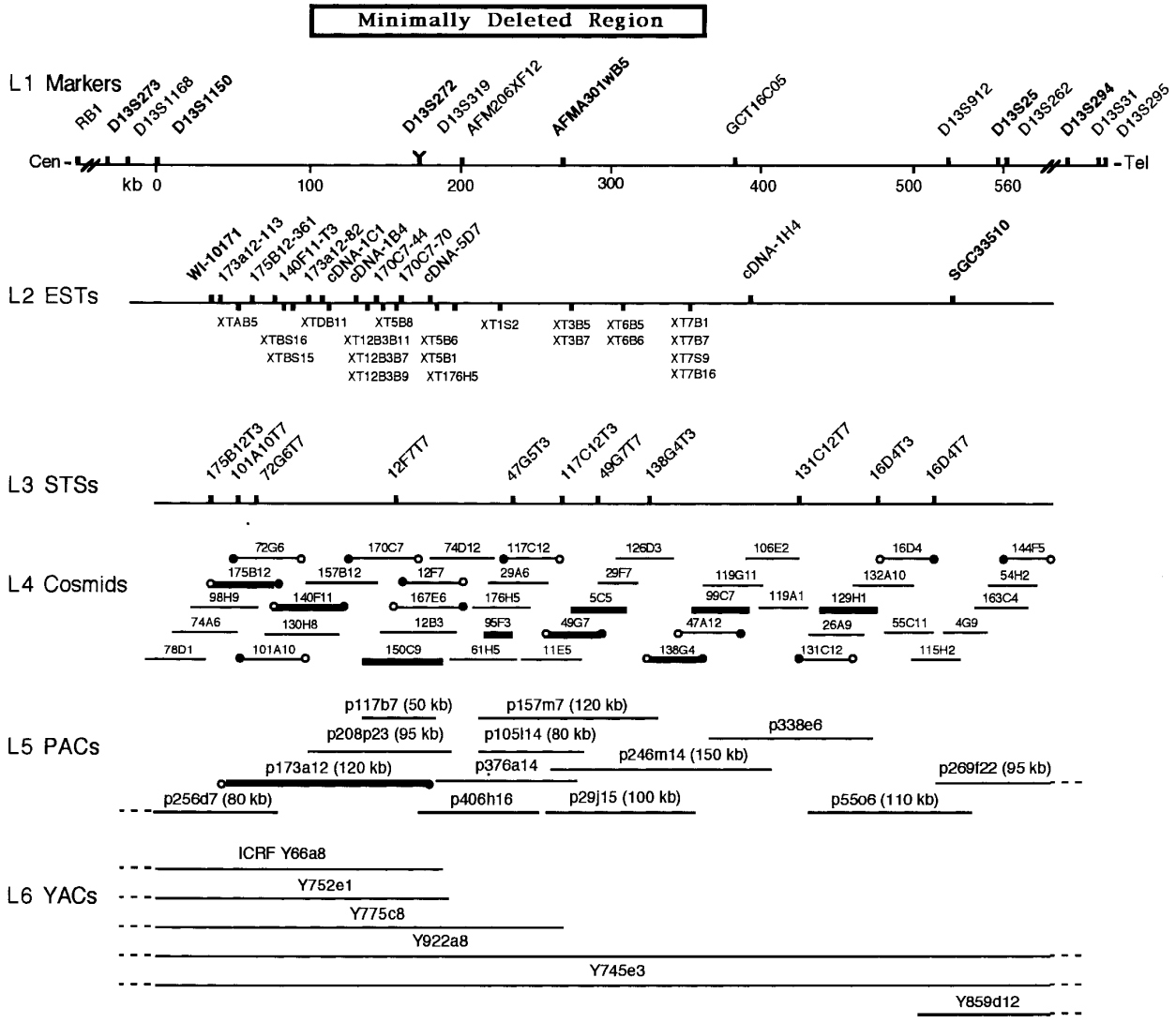

FIG. 1. Physical map of the CLL candidate gene region in 13q14. Map elements are shown in six layers (L1–L6) in the centromere (cen) to telomere (tel) direction. L1, markers from public databases. The markers used in deletion mapping are shown in boldface. The scale (in kb) was determined from clone overlaps. L2, ESTs. Shown in boldface are ESTs corresponding to sequences in public databases. L3, STSs developed from T3 and T7 insert ends of cosmids. L4, cosmids from the chromosome 13-specific cosmid library LA13NC01. The map shows a spanning path of cosmids between D13S1150 and D13S25. Heavy bars indicate cosmids used to generate probes for quantitative Southern blot analysis. Filled and open circles on cosmid bars identify T7 and T3 ends, respectively. L5, PAC contig assembled from clones in segments RPCI1 and RPCI3 of the human PAC library. Dashes at the ends of PACs indicate clones extending outside from the region. Clone lengths are drawn to approximate scale. L6, YACs from CEPH or ICRF libraries. The minimal region of deletion in CLL is shown on top. For further details see text.

by guanidine isothiocyanate extraction (Chirgwin *et al.*, 1979) and with the PolyATtract mRNA Isolation system (Promega, Madison, WI), respectively, from two CLL cell lines (Kawata *et al.*, 1993; and G. Inghirami, unpublished), three CLL samples without detectable 13q14 abnormalities, a lymphoma cell line (BJAB), and human tonsil and spleen specimens. To control for equal RNA loading and transfer, the blots were hybridized with a MAX cDNA probe (Gu *et al.*, 1993). In addition, expression profiles were determined by RT-PCR assays. For this purpose, cDNA was first sythesized from 1 μg of total RNA that was incubated in a 50-μl reaction volume with Superscript II RNAase H$^-$ reverse transcriptase (Life Technologies) under the conditions specified by the manufacturer. The cDNA products in 2 μl of the first reaction were then amplified by PCR by performing 30 cycles of denaturation (10 s at 94°C), annealing (30 s at 52–58°C), and extension (30 s at 72°C) in a 9600 DNA thermal cycler (Perkin–Elmer Corp., Norwalk, CT).

RESULTS AND DISCUSSION

Physical Mapping

A comprehensive physical map spanning the CLL candidate gene region, which displays all of the hybridization relationships used for map assembly (see Materials and Methods), is posted on the Columbia Genome Center homepage (http://genome1.ccc.columbia.edu/~genome/). This map includes a contig of 11 YACs spanning the region, 13 PACs in a single contig representing threefold PAC coverage, and 86 contiguous cosmid clones representing sixfold cosmid coverage. Here,

TABLE 1

Name	Primer	Primer sequence	Size (bp)
		(A) ESTs	
173a12-113	173a12-113F	TTACTGCTGCATCAAGAAC	137
	173a12-113R	ATGGTGTGTAGACACTGC	
175B12-361	175B12361F	GAATATAAGAGATTGAGTGC	153
	175B12361R	ACAAGTGACAAACTTCAGC	
140F11-T3	140F11T3F	CTGCACCTGGCCTTAACC	141
	140F11T3R	TGTTTTTGTCAGAATTTGACC	
173a12-82	173A1282F	AAGAAAGTGACCTGTGGC	153
	173A1282R	GTATATGTTGGGGATGGC	
cDNA-1C1	1C1F	AGATCATCTGAGTAGCAAC	188
	1C1R	TCCATGGAGTTCCTGAAG	
cDNA-1B4	1B4F	ACCTGTCCGAGAGTATAG	277[a]
	1B4R2	AGGTAAGAATTGCTGAGG	
170C7-44	170C7-44F	GCCATGATCCTGAATGTG	308
	170C7-44R	AGCCTCCTGAGTAGTAAG	
170C7-70	170C7-70F2	TTGAGTGCTTACTGTGTG	104
	170C7-70R2	TCAAAGTGTCGTATCTGG	
cDNA 5D7	5D7F	GCTCTTCGACCGTTTGG	183
	5D7-1B	TTCAGAGCTGAGGGTAAC	
cDNA-1H4	cDNA-1H4F2	TGACAGTCACACGGCTTC	188
	cDNA-1H4R	AATGGAGACTCAGGAAAGC	
		(B) STSs	
175B12T3	175B12T3F	AATGCCACGTGTACCCCA	249
	175B12T3R	TTCGCCTGCCTGTAATCC	
175B12/1	175B12/1F	TGAGAACTGACTAAACTACTG	198
	175B12/1R	CTGCTGAGGTGATCATCTC	
101A10T7	101A10T7F	CACAAACGCAATGCTAGG	363
	101A10T7R	TGTTGCAAGAAGGCACTG	
72G6T7	72G6T7F	TGATATTACGCATGGCACA	425
	72G6T7R	TGAGTTTACAGTGAGCCA	
12F7T7	12F7T7F	GCTTGGTCCGGTTTTCTG	210
	12F7T7R	TTCAGCAACTGCAGCAGG	
117C12T3	117C12T3F	ATGTAAGGTAAGTGTGCTC	224
	117C12T3R	TTCAGGCCGTGACATTAG	
49G7T7	49G7T7F	TCTCAGTAACAAGACCAC	275
	49G7T7R	ACCCTGTGATGTTCATAG	
138G4T3	138G4T3F	CCCGGTCACAATGGTCTG	70
	138G4T3R	AGTACCTGCATCGCCTTTG	
131C12T7	131C12T7F	GCAGCCTAAAGTGAGAATG	155
	131C12T7R	TTCTCTCTGCTGGCTGAG	
16D4T3	16D4G3F	TGTAGTCAAGTTTATGACCG	140
	16D4T3R	GAACTAATCCAGACAAAGAG	
16D4T7	16D4T7F	GTAGGAAGGCCAGGGCTC	240
	16D4T7R	GCTCTGGCAGTGATCTGTG	
47G5T3	47G5F	ATTTTCCTGGAGGAATGG	178
	47G5R2	TTGCTCTGGTACTTTGAC	

[a] Corresponds to RT-PCR fragment rather than genomic fragment.

only a summary version of this map is presented (Fig. 1), due to space constraints.

The correlated map elements are displayed in six layers (L1–L6; Fig. 1), and the minimal region of deletion defined from examined CLL cases (see below) is shown on top. L1 shows the order of 12 genetic markers from public databases and also a map scale (in kilobases) for all layers, which was derived from estimates of the extent of overlaps between cosmid and PAC clones, after measuring insert lengths by pulsed-field gel electrophoresis. Thus, in addition to the order of markers in L1, which is consistent with that reported previously (Brodyanskii *et al.*, 1995; Bullrich *et al.*, 1996), the distances between markers were determined quite accurately, since each marker corresponds to a unique set of cosmids and PACs in L4 and L5. Markers D13S272, AFMA301wB5, GCT16C05, and D13S25 were positioned on the map not only by PCR analysis, but also from the results of sample sequencing of cosmids 170C7, 49G7, 47A12, and 54H2, respectively.

The positions of 32 expressed sequences are shown in L2. Of these, 4 cDNAs (1C1, 1B4, 5D7, and 1H4) were first selected with ICRF YAC66a8 and PAC 246m14 then backmapped to cosmids in L4, while 6 ESTs (173a12-113, 175B12-361, 140F11-T3, 173a12-82, 170C7-44, and 170C7-70) corresponding to sequences in dbEST were identified by analysis of sample sequencing data (PCR primers for these 10 ESTs are listed in Table 1A). Two additional ESTs, shown in L2 in boldface, and cDNA 1H4 (see Table 2) correspond to sequences identified in the NCBI Transcript Map (Schuler *et al.*, 1996). The remaining sequences, all with the prefix XT, are trapped exons.

L3 shows the positions of 11 STSs, which were generated from sequencing data of cosmid insert ends and used to enhance the resolution of the map (PCR primers for these STSs are listed in Table 1B).

L4 shows an uninterrupted contig of 42 cosmids, a subset of which (heavy bars) was used to generate probes for deletion mapping.

An uninterrupted PAC contig covering an estimated physical distance of 560 kb between markers D13S1150 and D13S25 is shown in L5.

A subset of YACs used in map construction is shown in L6, to provide a reference to other published maps of the region (Brodyanskii *et al.*, 1995; Bullrich *et al.*, 1996) and Whitehead human STS vs YAC database.

Allelic Loss Analysis

To define a minimal region of deletion in CLL, paired normal and tumor DNA samples from 57 patients were tested for loss of heterozygosity with 17 markers within the D13S273–D13S294 interval. Five of these markers were polymorphic microsatellites (D13S273, D13S272, D13S319, AFMA301WB5, and D13S294) that were used for PCR analyses, while 12 DNA fragments from mapped cosmids (see Figs.1 and 3) were used as hybridization probes to determine allelic dosage by quantitative Southern blotting (see representative results in Fig. 2).

Allelic losses in the CLL region were observed in 28/57 (49%) of the cases, while the DNA from the remaining tumors did not exhibit a detectable deletion or alteration at 13q14. In the subset of cases with a deletion, monoallelic loss was observed in 23/28 (82%) of the tumors, while in 5/28 (18%) of the specimens the loss was biallelic. The informative data from these analyses are summarized in Fig. 3 (3 and 7 cases with biallelic and monoallelic deletions throughout the region, respectively,

TABLE 2

EST	Source	Cosmid localization	Database homologies[a]	Expression pattern[b] RT-PCR	Expression pattern[b] Northern (kb)
WI-10171	Human gene map	74A6 98H9	T87218 F09988 D19914	$+^{(1-6,8)}$	ND
173a12-113	Sequencing	74A6 98H9	N39807	$+^{(1-8)}$	—
175B12-361	Sequencing	74A6 98H9 175B12	R92258	$+^{(1-8)}$	—
140F11-T3	Sequencing	98H9 72G6 175B12 101A10 140F11	H48685 H48516	$+^{(1-8)}$	—
173a12-82	Sequencing/selection	101A10 130H8 140F11	T75567	$+^{(1-8)}$	—
cDNA-1C1	Selection	130H8 157B12	T91414 H43386 H48671 T55397	$+^{(2-8)}$	—
cDNA-1B4	Sequencing/selection exon trapping	175B12 130H8 157B12	N25204 D62401 D62413	$+^{(1-8)}$	1.3, $1.9^{(1-4)}$
170C7-44	Sequencing selection	157B12 170C7 150C9	F07317	$+^{(3,5,7,8)}$	—
170C7-70	Sequencing/selection exon trapping	157B12 170C7 150C9	R08323 R93227 H64330 R89810	$+^{(1-5,7,8)}$	$1.2^{(1-4)}$
cDNA-5D7	Selection	170C7 150C9 167E6 12B3	—	$\pm^{(2-7)}$	—
cDNA-1H4	Selection	99C7	SGC32580	$+^{(3)}$	ND
XT5B8	Exon trapping	170C7 150C9	—	$+^{(3,6-8)}$	—
XT1S2	Exon trapping	95F3 29A6	—	$+^{(3)}$	—
XT3B5	Exon trapping	49G7	—	$+^{(3,6-8)}$	—
XT3B7	Exon trapping	49G7	—	$+^{(3-8)}$	—
XT6B5	Exon trapping	29F7	—	$+^{(1,5,6-8)}$	—
XT6B6	Exon trapping	29F7	—	$+^{(1,3-8)}$	—
XT7B1	Exon trapping	138G4	—	$+^{(1-8)}$	—
XT7B7	Exon trapping	138G4	—	$+^{(1,3-8)}$	—
XT7S9	Exon trapping	138G4	—	$+^{(3,4,6,7)}$	—
XTAB5	Exon trapping	175B12	—	$+^{(1-8)}$	—
XTBS16	Exon trapping	74A6 175B12 130H8	—	$+^{(1,3-8)}$	—
XTBS15	Exon trapping	175B12 130H8	—	$+^{(1-5,7)}$	—
XTDB11	Exon trapping	130H8 157B12	—	$+^{(1-5,7)}$	—
XT7B16	Exon trapping	138G4	—	$+^{(3,6,7)}$	—
XT12B3B7	Exon trapping	12B3	—	$+^{(3)}$	—
XT12B3B9	Exon trapping	12B3	—	$+^{(3)}$	—
XT12B3B11	Exon trapping	150C9 12B3	—	$+^{(3)}$	—
XT176H5B1	Exon trapping	150C9 176H5	—	$+^{(1-8)}$	—
XT5B1	Exon trapping	150C9	—	$+^{(1,3-8)}$	—
XT5B6	Exon trapping	150C9	—	$+^{(1,3-8)}$	—

Note. ND, not determined.

[a] >80% identities are reported.

[b] Origin of RNAs tested: 1, 2, and 6–8, B-CLL cell lines and cases, respectively; 3, B-lymphoma cell line (BJAB); 4, spleen; 5, tonsil. For samples 5–8 only total RNA (and not poly(A)$^+$-selected RNA) was analyzed in Northern blot.

were not included). The cases shown (Fig. 3) can be classified into two groups. In the first group (9 cases with monoallelic and 2 with biallelic deletions), losses were detected in consecutive loci, suggesting elimination of a single DNA segment in each case. In contrast, a mosaic pattern of losses at noncontiguous loci was observed in a second group of tumors with monoallelic deletions (7 cases), suggesting that deletions, potentially involving DNA rearrangements, had occurred.

Based on the analysis of cases in the first group, the

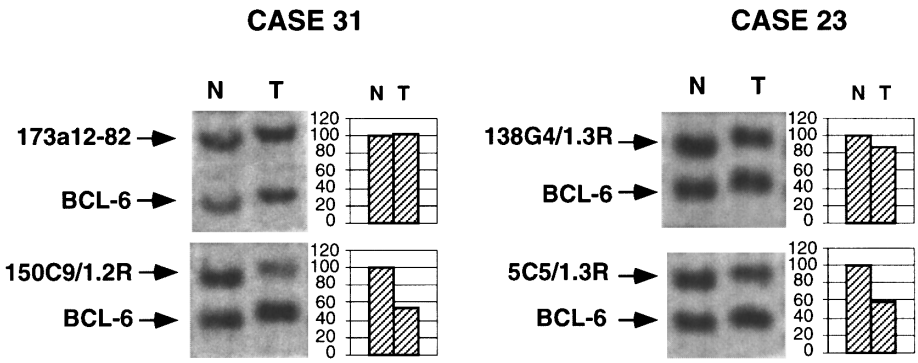

FIG. 2. Representative results of allelic dosage analysis performed by quantitative Southern blot hybridization. The results shown are taken from the analysis of CLL cases 31 and 23, which allowed the definition of the centromeric and telomeric border of the minimally deleted region, respectively. The tested probe (see Materials and Methods, *Allelic loss analysis* for a description of each probe used for the analysis) was cohybridized with a reference probe (BCL-6) onto filters containing *Eco*RI-digested genomic DNA derived from normal/tumor tissue pairs. The signal intensities were measured with a PhosphorImager and the values obtained with the test probe were normalized against those obtained with the reference probe. Normalized signals corresponding to the normal sample were considered 100% and normalized signals corresponding to the tumor lanes were expressed as percentage of the normal (see Materials and Methods, *Allelic loss analysis* for the description of the probes used for the analysis and for deletion scoring criteria). The position of the probes used is illustrated in Fig. 3.

borders of minimal deletion were assigned as follows: Centromerically, the segment identified by probe 173a12-82, which had been mapped to cosmid 140F11, was retained in case 31, while the entire region detectable by all of the more telomeric markers was deleted (see Fig. 2). The position of the centromeric border is consistent with the results from cases 20 and 23, which have lost the segment detectable by probe 173a12-82, but have retained the sequence recognized by marker 140F11-T3, which is included in the same cosmid (140F11). Telomerically, the border was defined by marker 138G4/1.3R, which was retained in case 23 (see Fig. 2).

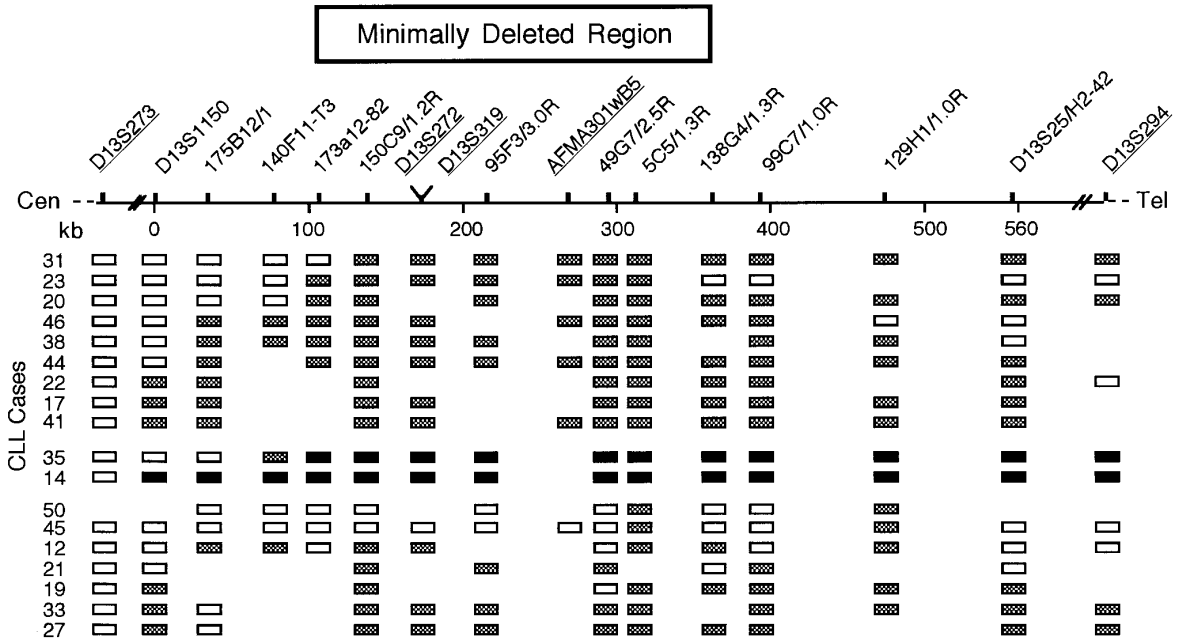

FIG. 3. Mapping the CLL minimally deleted region in 13q14. Shown are CLL cases that lost only a subset of the chromosome 13 markers tested and therefore are useful for the definition of the region of minimal deletion. The markers used (see Materials and Methods and Fig. 1) are indicated on top and include probes for Southern analysis and microsatellite markers. Numbers following the marker names indicate the sizes of *Eco*RI restriction fragments (R). They are distributed over a 1-Mb region from D13S273 (centromeric) to D13S294 (telomeric). Tumor cases are indicated by number on the left. The results of allelic loss analysis are represented with squares of three types: open, indicating retention; filled, indicating biallelic loss; and gray, indicating monoalleic loss. Loss in 11 of the cases (top to bottom, patient 31 to 14) appears to be continuous, while it is mosaic in the remaining tumors. Gaps are due to the fact that, in order to save the limited amounts of tumor DNA available, after an initial screening with a common set of probes, each case was studied further using a subset of probes chosen as the most appropriate to refine the deletion borders for that particular case. The pattern of allelic loss in patients 31 and 23 defines a <300-kb minimally deleted region flanked by, but not including, markers 173a12-82 and 138G4/1.3R.

Results consistent with the assignments of borders were obtained by using a subset of the markers and performing allelic loss analyses on an additional panel of 99 CLL tumors, 56 of which exhibited deletions (not shown). The size of the minimal region of deletion is <300 kb. This estimate, derived from the physical map, is based on the correspondence of the deletion to a spanning path of nine cosmids, considering that each cosmid contributes on average a nonoverlapping length of 30 kb (see Fischer *et al.,* 1994).

Identification of Transcribed Sequences

Toward the isolation of the putative CLL-associated tumor suppressor gene, we identified transcribed sequences within the D13S1150–D13S25 interval, which includes the defined region of minimal deletion, using several approaches. In addition to exon trapping and direct cDNA selection using immobilized YAC or PAC DNAs (see Materials and Methods), we performed sample sequencing of cosmids and PACs spanning the region and searched dbEST for homologies. ESTs from the NCBI human gene map (release of December 1, 1996) (Schuler *et al.,* 1996) and from the Whitehead Institute for Biomedical Research/MIT Center for Genome Research were also backmapped in the CLL candidate region. This was accomplished by hybridizing probes corresponding to these transcripts to colony filters of the 13q14 clone set and by performing PCR analyses on cosmids and PACs representing a spanning path through the region.

A total of 32 transcribed sequences were identified. For each sequence, Table 2 summarizes the method of identification, map position, pattern of expression in selected B-cell-derived RNAs (including three samples from CLL patients without a deletion at 13q14), and homologies to known ESTs. RT-PCR analyses with specific primers showed that 31/32 of these sequences were transcribed in the panel of B-cell tissues and tumor RNAs. However, only 2 of these sequences yielded a positive hybridization signal by Northern blot analysis of a panel of RNAs representative of normal and neoplastic lymphoid tissues (see Table 2), suggesting that most of the transcripts corresponding to the probes are expressed at low levels in the tissues tested. Of the expressed sequences identified, only 1C1 was found to be homologous to a known gene (3′ untranslated region of gene B94; Genebank Accession No. M92357).

CONCLUDING REMARKS

The rich set of uninterrupted clone contigs and large number of landmarks in the presented high-resolution physical map, which spans a <300-kb region of minimal deletion in CLL and allows reliable estimates of physical distances across the region, will be useful in establishing accurate deletion boundaries in any additional analyses of tumor samples to narrow the region further.

Previous studies have assigned various regions as candidates for the 13q14 CLL-associated tumor suppressor gene. Initially, a relatively large region telomeric to RB1 and centered around the D13S25 locus was proposed (Brown *et al.,* 1993). Subsequently, various studies based on LOH analysis of CLL panels refined further the limits of the minimal region of deletion to a 0.7- to 1-Mb interval bordered by RB1-D13S31 (Chapman *et al.,* 1994), RB1-D13S25 (Liu *et al.,* 1995), or D13S272 and D13S295 (Devilder *et al.,* 1995). A more recent study (Bullrich *et al.,* 1996) suggested as the candidate region a 550-kb segment bracketed by markers AFM206xF12 and D13S25. Our results allow further restriction of the minimally deleted region, which is either included in its entirety within some segments assigned previously (Brown *et al.,* 1993; Chapman *et al.,* 1994; Liu *et al.,* 1995) or is partially overlapping with others (Devilder *et al.,* 1995; Bullrich *et al.,* 1996). This partial overlap is not surprising considering that some of the markers used in these sudies were different and that to some extent the limits of the deletions may vary stochastically among various cohorts of patients. However, the partial overlap of the deletion consensus in our versus other studies can be used to pinpoint the crucial genomic segment believed to include the CLL-associated gene, or part of it. Combining the results of all the published CLL cases, the consensus region would be defined by four overlapping cosmid clones (see Fig. 1) spanning <160 kb and bordered by marker AFM206xF12 (centromeric) and probe 138G4/1.3R (telomeric).

An additional important use of our map will be the accurate positioning of further ESTs, although many candidate exons have already been identified. Considering that the average distribution of genes in the human genome is 1 per 30 kb (see Miklos and Rubin, 1996), the number of ESTs in the CLL candidate region (1/13 kb; 23/300) is quite high and some of them are likely to belong to the same transcription unit. Thus, mutational analysis of DNA from cases without biallelic deletion can be initiated with these available candidate sequences.

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