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Long-distance restriction mapping of the proximal long arm of human chromosome 21 with *Not* I linking clones

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ABSTRACT **Human chromosome 21 is the smallest of the 22 autosomes and 2 sex chromosomes. Hybridization of the human repetitive sequence *Alu* to pulsed-field gel-fractionated *Not* I-digested genomic DNA from a human–mouse hybrid cell line containing chromosome 21 as the sole human component identified chromosome 21 *Not* I restriction fragments. A *Not* I restriction map of regions of the chromosome was constructed, by identifying neighboring *Alu* bands with *Not* I linking clones. This approach simplifies the task of physical mapping and avoids ambiguities in *Not* I fragment assignments that arise from gel-to-gel mobility variations. A contiguous map was constructed with six *Not* I linking clones that covers at least the proximal one-third of the long arm of chromosome 21 and spans 20 megabases. A more detailed restriction map revealed 11 likely CpG islands in this region and localized 11 additional DNA markers.**

Chromosome 21, the smallest human chromosome (1), is estimated to be about 50 megabases (Mb) long. Linkage analysis suggests that familial Alzheimer disease can be caused by a defect in a gene on the proximal long arm of the chromosome (2–4). Trisomy of chromosome 21 results in Down syndrome (5). Genes causing familial amyotrophic lateral sclerosis and progressive myoclonus epilepsy (Unverricht–Lundborg type) are also linked to chromosome 21 (6, 7). Construction of a physical map of this chromosome is thus of great interest as a model for more arduous mapping projects on larger chromosomes, and for its use in finding these disease genes.

Several approaches have been used to construct physical maps of chromosome 21 (8–12). Gardiner *et al.* (9) distributed >60 DNA markers into 13 regions on the long arm of the chromosome by using somatic cell hybrids containing limited regions of the chromosome. Most markers were also used as hybridization probes to detect fragments generated with restriction enzymes that cut infrequently, after fractionation by pulsed-field gel electrophoresis (PFG; ref. 13). Cox *et al.* (11) and Burmeister *et al.* (12) constructed a long-range physical map of 30 markers by using radiation hybrids. Some of the same markers were used to construct restriction maps totaling several megabases.

A linking clone is a short genomic clone containing a cleavage site for a particular rare-cutting enzyme (14). *Not* I linking clones contain a *Not* I restriction site and identify two adjacent genomic *Not* I fragments when used as hybridization probes. Linking clones have been used to localize human disease-related genes (15, 16). The potential effectiveness of linking clones in map construction has been stressed (17), but the scarcity of these clones has limited most applications to confirming or finishing maps assembled predominantly by other methods. Here, we report the *de novo* application of these clones for the construction of a long-distance physical map and demonstrate the power of this approach.

MATERIALS AND METHODS

Cell Lines, DNA Markers, and Libraries. A human–mouse hybrid cell line, WAV17 (18), obtained from F. H. Ruddle (Yale University), with chromosome 21 as the sole human component was the genomic DNA source for the chromosome 21 restriction map. Cell lines that had lost chromosome 21 or retained only a single copy were clonally obtained by rescreening WAV17 and used as a control mouse cell line and a WAV17 standard cell line, respectively. A mouse cell line, A9, was also used as a control. Human cell lines, Namalwa, HeLa, and Raji, with a pair of normal chromosomes 21 were used to confirm the physical map. GM06136 with a 10;21 translocation resulting in monosomy for 21q22.3–qter and GM09552 with a 3;21 translocation resulting in trisomy for 21pter–q21 were purchased from the Human Genetic Mutant Cell Repository and used for regional mapping of linking clones.

DNA markers (clone names) were: *Alu* (Blur8) (19), *APP* (pAZ-10) (20), *D21S1* (pPW228C) (21), *D21S4* (pPW233F) (21), *D21S8* (pPW245D) (21), *D21S11* (pPW236B) (21), *D21S13* (G21RK) (22), *D21S16* (pGSE9) (23), *D21S17* (pGSH8) (23), *D21S26* (26C) (24), *D21S46* (SF85) (25), *D21S52* (pPW511-1H) (26), *D21S82* (Fr8-77) (27), *D21Z1* (L1.26) (28), and L1 (pUK20A) (29). Two human chromosome 21-specific λ Charon 21A libraries (30) used for isolation of *Not* I linking clones were purchased from the American Type Culture Collection.

Chromosome 21 *Not* I Linking Clones. A *Not* I linking library was constructed by *supF* rescue from a Charon 21A (W_{amber},E_{amber}) library containing *Hin*dIII inserts prepared from flow-sorted human chromosome 21 essentially as described (31). Fourteen unique *Not* I linking clones isolated from this library are named with the prefix LL. Also used were two clones previously isolated (31); these are named with the prefix LA. Six more clones were obtained by rescreening the original libraries with noncontiguous clones (31). Their names contain the suffix LP or SP. G21RK (*D21S13*) was also included as a *Not* I linking clone.

PFG. Genomic DNA, prepared in agarose, was digested with restriction enzymes, fractionated with a Bio-Rad (CHEF-DRII) or a Pharmacia LKB (Pulsaphor) PFG appa-

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Abbreviations: Mb, megabase(s); PFG, pulsed-field gel electrophoresis.

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ratus, and hybridized as described (32–34). DNA samples prepared at 0.5×10^7 cells per ml were used for size estimation of *Not* I fragments, because DNA migration in PFG is very sensitive to DNA concentration (31). Size standards were bacteriophage λ concatemers (35), *Saccharomyces cerevisiae* YNN295 chromosomal DNA and *Schizosaccharomyces pombe* chromosomal DNA (36). Probes with repetitive sequences were preincubated for 2 hr at 42°C, usually with human placental DNA (200 μg/ml) present. Autoradiography employed either x-ray films or a bioimage analyzer (Fujix BAS2000; Fuji Film, Tokyo).

Regional Mapping. Genomic DNA of GM06136 and GM09552, cell lines aneuploid for parts of chromosome 21, was prepared, digested with *Eco*RI, electrophoresed in 0.6% agarose, transferred to a nylon membrane (33), and hybridized simultaneously with one half-fragment of a *Not* I linking clone and a mapped DNA marker, *D21S17* (9), present in two copies in the two cell lines as an internal reference. The signal intensity was used to estimate the copy number of the clones. Clones with two copies in GM06136 and three in GM09552 were assigned to pter–q21; with two in both cell lines, to q21–q22.3; and with one in GM06136 and two in GM09552, to q22.3–qter.

RESULTS

***Alu* Banding and Linking-Clone Mapping.** Linking clones from opposite ends of a *Not* I fragment detect the same-sized *Not* I fragment in hybridizations to *Not* I-digested genomic DNA fractionated by PFG. Two clones that appear to share the same *Not* I fragment tentatively assign these *Not* I sites as neighbors on the map. A complete library of *Not* I linking clones potentially allows the construction of a *Not* I restriction map, even of a complex genome. A key step is accurate sizing of the *Not* I fragments. In practice, because of ambiguities in PFG sizing, it is often hard to conclude that two linking clones have detected the same *Not* I fragment.

Fig. 1 shows *Alu* probing of PFG-fractionated *Not* I-digested genomic DNA from a human–mouse hybrid cell line (WAV17) with chromosome 21 as the sole human component (Fig. 1). *Alu* hybridization to a set of PFG fractionations at different conditions revealed 33 discrete bands. These total >40 Mb, which is >80% of the presumed size of chromosome 21. The intensity of *Alu* hybridization does not simply increase linearly with *Not* I fragment size. The strong deviation from this expected pattern reflects the nonrandom distribution of *Alu* sequences on the chromosome (37). Some faint bands originate from partial digests, but most do not, since they appeared as unique bands when probed with particular *Not* I linking clones or changed to intense bands when probed with particular *Not* I linking clones or changed to intense bands when probed with the repetitive sequence L1. All 33 *Alu* bands were derived from human chromosome 21, since genomic DNA of a cell line derived from WAV17 that had lost human chromosome 21 showed no bands. Some bands are composed of multiple DNA fragments. Thus, the total length of all the *Not* I fragments is >40 Mb.

*Hin*dIII (or *Eco*RI) inserts of the linking clones were cleaved with *Not* I, and the two *Hin*dIII–*Not* I (or *Eco*RI–*Not* I) half-linking fragments, L (larger) and S (smaller), were used separately as hybridization probes. Analyses with 46 half-linking fragments from 23 unique linking clones revealed that all of the *Not* I fragments detected, except one, corresponded to *Alu* bands, and 28 of the 33 *Alu* bands were identified with one or more of these half-linking fragments.

A number of *Alu* bands were detected by half-linking fragments from two different linking clones. This allowed us to construct a tentative *Not* I restriction map containing six *Not* I sites using six linking clones, G21RK, LA297LP, LL54, LL56, LL136, and LL152 (see Table 1). The results shown in Fig. 2 *A* and *B* indicate that *Alu* band 26 was shared by two half-linking fragments, LL136S and LL152L. In addition, *Alu* band 2 was shared by LA297LPS and LL54S; *Alu* band 8 by LL56L and LL152S; and *Alu* band 15 by G21RKS and LL56S (data not shown). *Alu* band 3 was shared by three half-linking fragments, LA297LPL, LL54L, and LL136L (Fig. 2 *C–E*), indicating that this band is composed of multiple fragments. These analyses (Table 1) suggest that the six linking clones are arranged either as G21RK–LL56–LL152–LL136–LL54–LA297LP or as G21RK–LL56–LL152–LL136–LA297LP–LL54, and *Not* I fragments are in the order >5.7 Mb–0.72 Mb–1.90 Mb–0.35 Mb–3.2 Mb–4.8 Mb–3.2 Mb. To confirm this we must still exclude the possibility that these probes detected different DNA pieces with coincident sizes.

Regional mapping of the six linking clones employed quantitative Southern blot hybridization on two human cell lines aneuploid for parts of chromosome 21. This allocated linking clones to one of three regions, pter–q21, q21–q22.3,

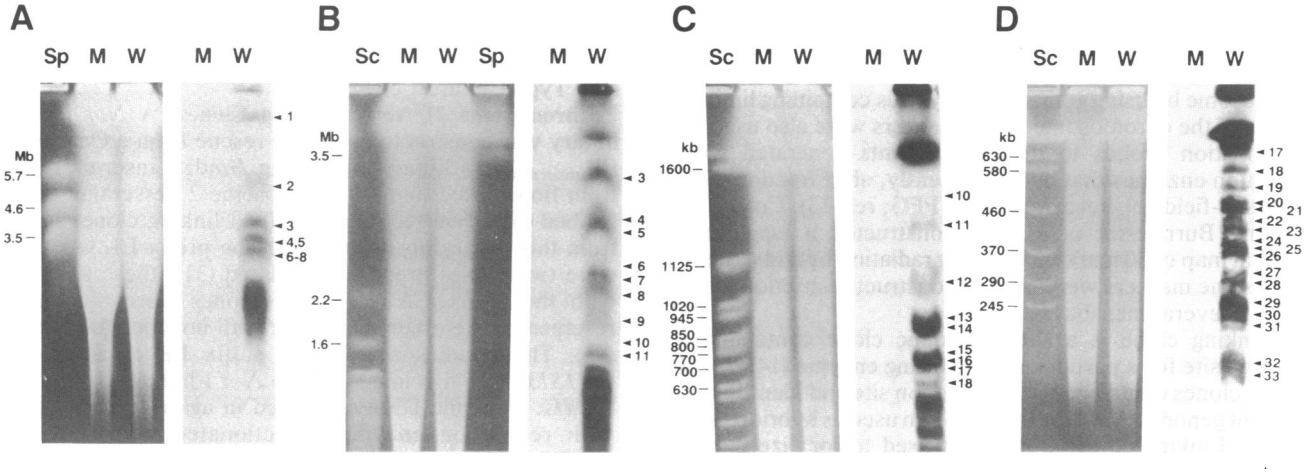

FIG. 1. Detection of *Not* I fragments of human chromosome 21 by *Alu* hybridization. *Not* I-digested genomic DNA of a human–mouse hybrid cell line, WAV17 (lanes W), containing chromosome 21 as the sole human component and a control mouse cell line (lanes M) was fractionated by PFG with *Sch. pombe* (lanes Sp) and *S. cerevisiae* (lanes Sc) chromosomal DNA as size standards and stained with ethidium bromide (left lanes in each run). The fractionated DNA was transferred to membranes and hybridized with *Alu* (right lanes in each run). A total of 33 discrete bands were detected in lanes W (*Alu* bands), numbered as indicated. PFG fractionations used a ramped pulse time from 1800 to 3600 sec in 0.6% agarose for 140 hr at 1.5 V/cm (*A*), from 300 to 900 sec in 0.8% agarose for 72 hr at 3 V/cm (*B*), from 60 to 120 sec in 1% agarose for 30 hr at 6 V/cm (*C*), and from 20 to 50 sec in 1.5% agarose for 24 hr at 6 V/cm (*D*). kb, Kilobases; Mb, megabases.

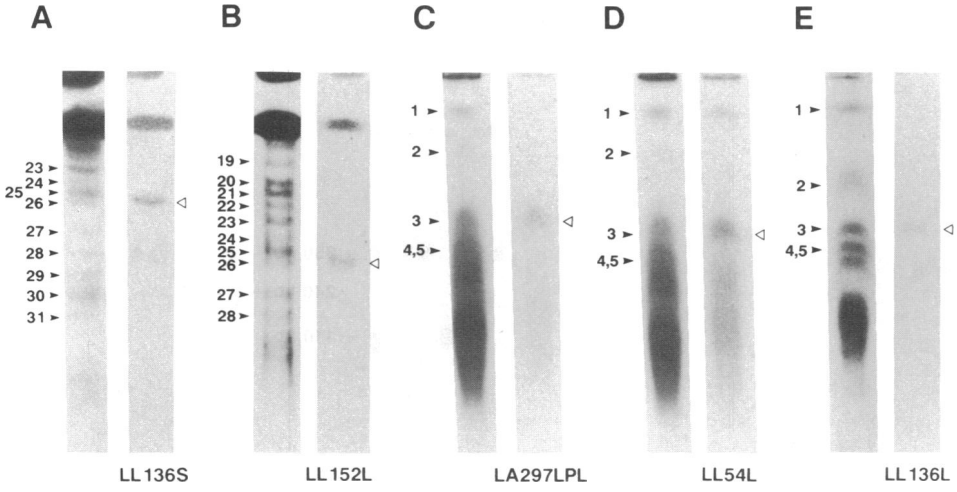

FIG. 2. *Alu* bands detected with half-linking fragments of *Not* I linking clones. WAV17 genomic DNA, digested with *Not* I and fractionated by PFG, was hybridized with *Alu* (left lanes) or with half-linking fragments LL136S (*A*), LL152L (*B*), LA297LPL (*C*), LL54L (*D*), and LL136L (*E*) (right lanes). Open triangles indicate detected signals. PFG fractionations used a ramped pulse time from 10 to 30 sec in 1.2% agarose for 20 hr at 6 V/cm (*A*), from 20 to 40 sec in 1.5% agarose for 30 hr at 6 V/cm (*B*), from 1200 to 2400 sec in 0.5% agarose for 120 hr at 1.5 V/cm (*C* and *D*), and from 1800 to 3600 sec in 0.6% agarose for 140 hr at 1.5 V/cm (*E*).

and q22.3–qter. G21RK, LL56, LL136, and LL152 were mapped in region pter–q21, and LA297LP and LL54 were mapped in region q21–q22.3 (Table 1). *D21S13*, a DNA marker derived from G21RK, has been mapped in the proximal region of the long arm of chromosome 21 (8–11). These facts indicate that the orientation is cen–G21RK–LL56–LL152–LL136–LL54(or LA297LP)–LA297LP(or LL54)–qter and that this mapped region is located in the proximal long arm.

Confirmation of the Physical Map. *Alu* band 3 was shared by three half-linking fragments. Thus it contains more than one *Not* I fragment. Bands 2, 8, 15, and 26 were shared by pairs of half-linking fragments, but some of these bands might still have been composed of multiple fragments, which could have invalidated our map. Two methods were used to confirm whether pairs of half-linking fragments were derived from the two termini of the same *Not* I fragment. The recognition sequence of *Not* I (GCGGCCGC) contains two CpG sequences potentially accessible to mammalian methylases. Methylated sequences are not cleavable by *Not* I, and methylation patterns are often different among cell lines. The first method was based on this potential polymorphism. The second was to see whether the *Not* I fragments detected by each pair of half-linking fragments shared the same spectrum of accessibility to other restriction nucleases with infrequent sites in mammalian genomes.

Two of the linking clones, G21RK and LL54, had polymorphic *Not* I genomic DNA digestions (Table 1). The *Not* I site in G21RK was not cleaved in the Raji cell line. The conclusion that G21RK is the neighbor of LL56 is consistent with the observation that a *Not* I fragment longer than 0.72 Mb was detected in Raji when probed with G21RKS or LL56S (Fig. 3). The alignment of LL136, LL54, and LA297LP was similarly determined, using polymorphism at the *Not* I site of LL54. Hybridization with LL136L and LL54L detected a 3.2-Mb *Not* I fragment in WAV17, but a longer fragment in the other cell lines; whereas in all of the cell lines tested, LA297LPL detected a 3.2-Mb fragment (data not shown). A 4.8-Mb fragment was detected with LL54S and LA297LPS in WAV17, but a longer fragment was observed in the other cell lines (data not shown). This indicates that LL136 and LL54, and LL54 and LA297LP, are neighboring, but LL136 and LA297LP are not (see Fig. 5), so the order of the *Not* I sites is LL136–LL54–LA297LP.

The adjacency of LL56 and LL152, and of LL152 and LL136, was confirmed by the second method. The 0.35-Mb fragment recognized by LL152L contains *Bss*HII, *Eag* I, and *Sac* II sites but not *Mlu* I and *Nru* I sites (Fig. 4*A*). An identical pattern of these sites occurred on the 0.35-Mb fragment detected with LL136S (Fig. 4*B*), indicating that LL152 and LL136 are neighboring. The results also reveal the existence of a cluster of rare-cutting enzyme sites between the two *Not* I sites, 0.11 Mb from LL152. Similarly, we confirmed the adjacency of LL56 and LL152, and found a *Bss*HII site between them. Using all 12 half-linking fragments of these six linking clones, we constructed *Not* I restriction maps in three human cell lines and a more detailed restriction map in WAV17 for five additional restriction enzymes with rare cutting sites (Fig. 5).

Alignment of Commonly Used DNA Markers. Hybridizations revealed that the DNA markers *D21S1*, *D21S4*, *D21S8*, *D21S11*, *D21S16*, *D21S26*, *D21S46*, *D21S52*, *D21S82*, *D21Z1*, and *APP* (β-amyloid precursor protein gene) were located in the mapped region shown in Fig. 5. The chromosomes 13- and 21-specific alphoid DNA marker *D21Z1* was detected on the >5.7-Mb *Not* I fragment (*Alu* band 1) but not on the 1.45-Mb *Nru* I–*Not* I fragment centromeric to G21RK (see Fig. 5). The possibility that *Alu* band 1 is composed of multiple fragments and *D21Z1* is not located on the map in Fig. 5 seems unlikely, because *Alu* band 1 has not been

Table 1. Characteristics of *Not* I linking clones used for map construction

		Not I cleavability in genomic DNA of cell line*				Cleavability in WAV17 genomic DNA by enzyme*					Fragment			
											Half-linking, kb		Genomic *Alu* band no. (Mb)	
Clone	Region	W	N	H	R	B	E	M	U	S	L†	S†	L†	S†
G21RK	pter–q21	+	+	+	−	+	+	−	−	+	5.2	3.0	1 (>5.7)	15 (0.72)
LA297LP	q21–q22.3	+	+	+	+	+	+	−	−	+	3.3	2.7	3 (3.2)	2 (4.8)
LL54	q21–q22.3	+	−	−	−	+	+	−	p	+	3.0	1.00	3 (3.2)	2 (4.8)
LL56	pter–q21	+	+	+	+	+	+	+	−	+	3.6	0.97	8 (1.90)	15 (0.72)
LL136	pter–q21	+	+	+	+	+	+	−	+	+	1.95	0.32	3 (3.2)	26 (0.35)
LL152	pter–q21	p	+	+	+	+	p	−	−	−	4.4	0.87	26 (0.35)	8 (1.90)

*p, partial; W, WAV17; N, Namalwa; H, HeLa; R, Raji; B, *Bss*HII; E, *Eag* I; M, *Mlu* I; U, *Nru* I; S, *Sac* II.
†L, larger; S, smaller.

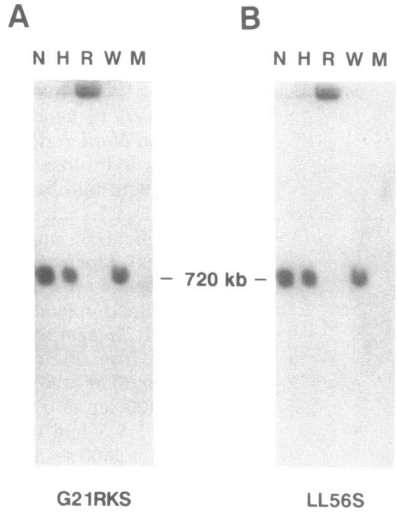

FIG. 3. Confirmation of the adjacency of LL56 and G21RK by *Not* I fragment length polymorphism. *Not* I-digested genomic DNA of three human cell lines, Namalwa (N), HeLa (H), and Raji (R), one human–mouse hybrid, WAV17 (W), and one mouse control cell line (M) was fractionated by PFG, transferred to a membrane, and hybridized successively with LL56S (*B*) and G21RKS (*A*). PFG was in 1% agarose for 17 hr at 6 V/cm with a ramped pulse time from 30 to 150 sec.

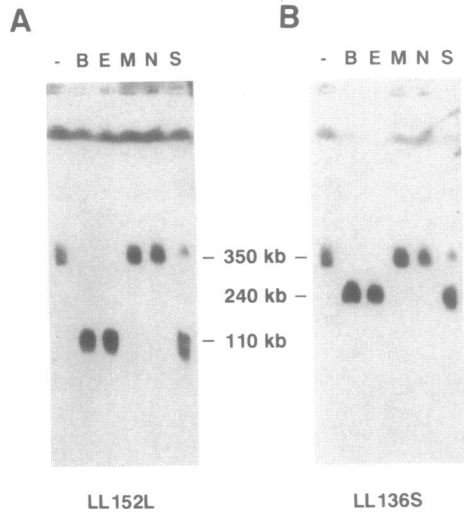

FIG. 4. Confirmation of the adjacency of LL152 and LL136 by double digestion. WAV17 genomic DNA, digested with *Not* I alone (−) or digested with *Not* I and another infrequently cutting enzyme [*Bss*HII (B), *Eag* I (E), *Mlu* I (M), *Nru* I (N), or *Sac* II (S)], was fractionated by PFG, transferred to a membrane, and hybridized successively with LL152L (*A*) and LL136S (*B*). PFG was in 1% agarose for 16 hr at 6 V/cm with a ramped pulse time from 10 to 50 sec.

detected with any other markers or half-linking fragments except *D21S16* and G21RKL. *D21S82* is located on the distal part of the map. A cDNA marker of *APP* was mapped on the distal of the two 3.2-Mb fragments (*Alu* band 3) and assigned to a small region distal to LA297LP. Four commonly used markers, *D21S1*, *D21S11*, *D21S13*, and *D21S16*, are known to be genetically linked with familial Alzheimer disease (2, 3). Our mapping indicates that the distance between *D21S1/S11* and *D21S13/S16* loci is at least 2.9 Mb.

DISCUSSION

Gardiner *et al.* (9) assigned chromosome 21-specific restriction fragments generated by a number of rare-cutting enzymes to various regions on the long arm of the chromosome. They identified 33 independent *Not* I fragments, using 52 probes. A number of fragment length discrepancies between their results and ours may be due to polymorphisms between the cells used. A comparison of our physical map and that of Cox *et al.* (11), constructed mainly by radiation-hybrid mapping, reveals that the order of the markers is identical, and relative distances between the markers are very similar.

Not I linking clones, once assembled into a long-distance contiguous map, are especially high-quality landmarks. Their orientation is defined by the two half-linking fragments, L and S. They can be used as probes in indirect end labeling to determine the distances of nearby gene loci. They allow the

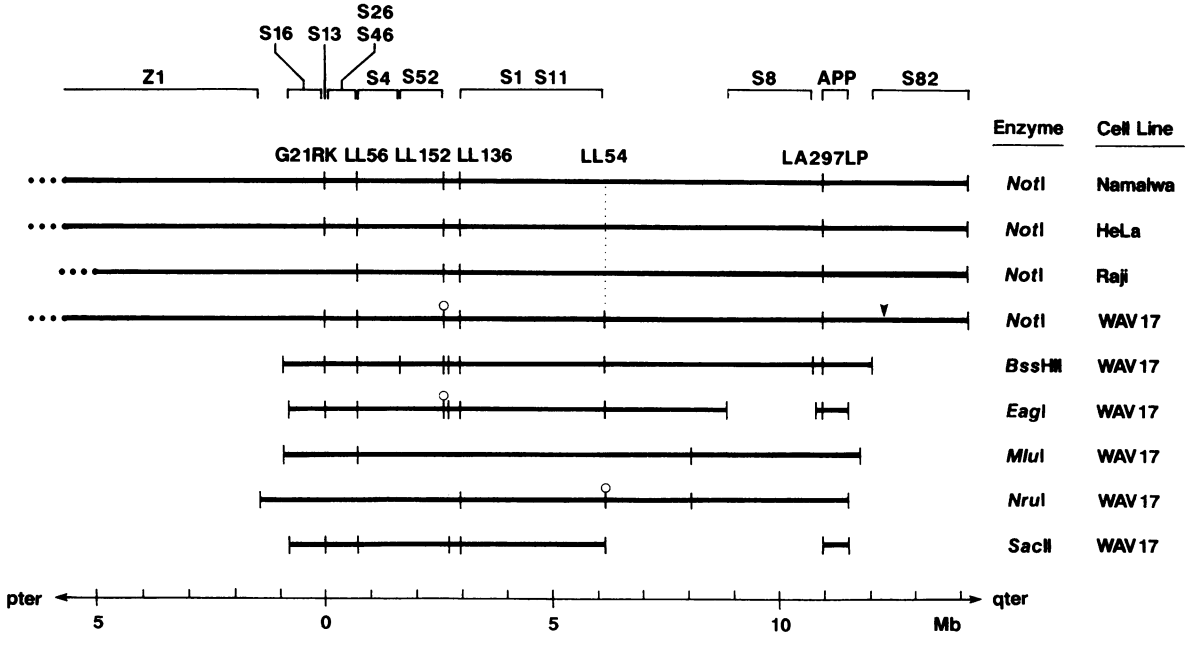

FIG. 5. Restriction map of the proximal region of the long arm of chromosome 21. DNA markers mapped in this region are shown at the top. Scales represent distances (Mb) from the *D21S13* marker. Restriction sites marked with open circles are cleaved partially (see Table 1). The *Not* I site of linking clone LL152 is not cleaved completely in WAV17, resulting in a faint band corresponding to *Alu* band 6. In some WAV17 lineages, a partially cleavable *Not* I site (arrowhead) is present in the 3.2-Mb *Not* I fragment distal to LA297LP.

systematic detection of chromosomal aberrations such as translocations, deletions, and gene amplifications, by *Not* I fragment length variation in genomic Southern hybridizations. For example, we have detected a translocation breakpoint in acute myeloid leukemia with t(8;21) by using a *Not* I linking clone from another region (38).

PFG allows the separation of DNA fragments up to megabases in size and provides a reasonably accurate estimation of these sizes. However, PFG mobilities are markedly affected by the concentration of DNA in the agarose sample blocks. This variation in mobility is especially noticeable when DNA from organisms with a large genome is fractionated. Errors can occur when *Not* I fragment sizes detected in separate PFG experiments are compared. In the present study, the *Not* I restriction map was constructed with help of *Alu* banding. The overall features of the pattern of *Alu* bands remained unchanged even when DNA was overloaded, although the resolution of the separation decreased somewhat. Knowledge of the alignment of *Alu* bands also facilitates the mapping of single-copy DNA markers simply by comparing their hybridization signals to known *Alu* bands. Eleven DNA markers shown in Fig. 5 were mapped in this way.

The physical map shown in Fig. 5 includes the sites for five additional infrequently cutting restriction enzymes, *Bss*HII, *Eag* I, *Mlu* I, *Nru* I, and *Sac* II. The cleavage patterns with these enzymes revealed 11 places where their cutting sites are clustered. These enzymes contain two CpG dinucleotides in their recognition sequences. Their clustered sites are probably in CpG islands. Further analysis of these loci will be interesting, since CpG islands are usually in the 5′ flanking regions of genes (39).

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