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## Integrated Radiation Hybrid and Yeast Artificial Chromosome Map of Chromosome 9p

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### Abstract

A panel of 93 radiation-reduced hybrids have been screened using PCR amplification and oligonucleotide primers for sequence-tagged sites (STSs) specific for 114 single-copy loci mapping to the short arm of chromosome 9. An x-ray dose of 6,000 rads gave an average retention frequency of approximately 23%. We have constructed a framework map containing 31 markers ordered by analyzing coretenation patterns, with support for the order greater than 1,000:1. In addition, we have placed the remaining markers which could not be mapped to a single interval with this support to a range of intervals on the framework map. The STS oligonucleotide primers used in the construction of the radiation hybrid (RH) map have been used to isolate and order yeast artificial chromosomes (YACs) assigned to 9p identified from the CEPH mega-YAC library. Eighty-nine STS markers have screened positive with at least one YAC. A total of 88 individual YACs (with an average size of 0.9 MB) have been placed on the map in a series of contigs and in some cases mapped cytogenetically by fluorescence in situ hybridization. Additionally, the YAC information has been used in conjunction with the RH framework placements to generate an integrated map containing 65 loci including 51 uniquely positioned markers, with an average resolution of 0.79 Mb.

### Key Words

Radiation hybrid  
Yeast artificial chromosome  
Chromosome 9p

### Introduction

The study and mapping of the short arm of chromosome 9 has become increasingly focussed following the localization of candidate tumor suppressor genes in several regions. Terminal deletions of 9p (distal to D9S1813 and between D9S263 and D9S1845) have been reported in several breast cancer cell lines using fluorescence in situ hybridization (FISH) mapping with a panel of yeast artificial chromosomes (YACs) from 9p and LOH studies in

lung cancer cell lines have shown the highest regions of deletion around D9S259 [1]. The CDKN2 gene which encodes an inhibitor (p16) of cyclin-dependent kinase is located on 9p21 [2, 3]. There is a considerable amount of data on the CDKN2 locus, supporting its association with many types of human cancer. Homozygous deletions of CDKN2 and CDKN2B have been described in a variety of tumors including leukemia, breast cancer, bladder cancer, glioma, ovarian cancer, melanoma, lung cancer and renal cancer [3–7]. Homozygous deletions in tumor cells

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occur less frequently than allelic loss and are associated with tumor suppressor inactivation, the main mechanism of inactivation in these candidate genes. The CDKN2 locus has also been linked with the familial form of melanoma [5, 8, 9]. Furthermore, the human gene encoding the cytoskeletal protein talin has been suggested as another potential tumor suppressor gene [10]. This chromosomal region is of additional interest due to the mapping of disease loci including hyperglycinemia (isolated nonketotic type 1) to 9p24–p23 [11] and venous malformations to 9p [12, 13]. More recently, the constitutional 9p syndrome was located between D9S267 and D9S286, and a sex determination gene was placed between SNF2L2 and D9S144 [1].

Several maps which include chromosome 9p as a component of genome-wide efforts have been published. These include genetic linkage maps based on meiotic recombination in the CEPH families [14–16], a Génethon-Cambridge University radiation hybrid (RH) map [17], a CEPH YAC map [18], an integrated sequence-tagged site (STS) YAC-RH map produced by the Whitehead Institute and Génethon [19] and a gene map produced by an international consortium [20]. We wished to exploit the information on these maps by firstly improving on the previously published RH map of chromosome 9p [21] with the addition of new markers on the framework map and subsequently refining the localization of these and previous markers. The second step would be to use this new and improved RH map as a basis for constructing a YAC minimal tiling path across the whole of the short arm of chromosome 9. Thirdly, the locations of markers based on YAC addresses together with data from a refined RH map should provide the basis for a robust integrated RH/YAC transcript map.

## Materials and Methods

### *Radiation Hybrids*

The RH panel used in this study had previously been constructed for chromosome 9 as described [21]. Briefly, the human donor somatic cell hybrid GM10611 was fused to the hamster cell line A23 [22, 23]. This line had previously been characterized by FISH using total hybrid DNA as a probe onto normal metaphase chromosome spreads [24] and was shown to contain the whole of chromosome 9 as the only cytogenetically detectable human material. Prior to fusion, GM10611 cells were exposed to 6,000 rads of x-rays. The hamster cell line deficient for thymidine kinase activity was added and fusion completed with polyethylene glycol [25]. Ninety-three independent RH clones were selected in complete DMEM plus HAT [26].

### *Growth of YAC and YAC DNA Preparation*

Yeast colonies were obtained from the HGMP, Hinxton, Cambridge, UK. Yeast was grown and DNA prepared as described [27]. In brief, yeast was grown in 5 ml UT medium (8 mg/ml Difco yeast nitrogen base, 0.1 mg/ml adenine HCl, 55 µg/ml tyrosine, 11 mg/ml Difco casamino acids, 20 mg/ml filter-sterilized dextrose) containing 50 µg/ml ampicillin for 2 days at 30°C with shaking and resuspended in 0.5 ml sorbitol solution (0.9 M sorbitol, 0.1 M Tris, pH 8, 0.1 M EDTA). Fifty microliters 1,000 U/ml lyticase and 50 µl 0.28 M 2-mercaptoethanol were added and the cells incubated at 37°C for 1 h in a shaking incubator, resuspended in 0.5 ml 0.5% SDS/100 mM Tris, pH 8/50 mM EDTA solution and incubated at 70°C for 20 min. After adding 80 µl 3 M KAc/1.8 M formic acid, incubation at 4°C and ethanol precipitating, the YAC DNA was resuspended in water. YAC DNA was cleaned by adding 7.4 M NH<sub>4</sub>Ac and ethanol precipitation and ethanol washing.

### *Polymerase Chain Reaction*

RH clones had been previously characterized by *Alu*-specific PCR for human content [21]. The presence or absence of single-copy loci in the RH or YAC panels was determined by PCR. Each marker was screened in duplicate. Unless otherwise indicated, all PCR reactions were performed using the following conditions: briefly, PCR reactions contained 300 ng DNA for RH or 2 ng DNA for YACs, 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 300 ng each primer, 1 U of Taq polymerase and water to a final volume of 50 µl overlaid with 50 µl of mineral oil to prevent evaporation. An initial cycle at 94°C for 1 min was followed by 30 cycles of denaturing at 94°C for 1 min, annealing at 55°C (D9S1210, D9S1224: 50°C; D9S161: 57°C; D9S43, D9S144, D9S259, D9S1948, CNTFR, GALT: 58°C; D9S1049E: 59°C annealing) for 1 min and extending at 72°C for 1 min. PCR reactions were carried out with 96-well microtiter plates on a Techne GeneE cycler. The 96-well 'microtiter format' was used with a Beckman 1000 Biomek robot to aliquot DNA for rapid PCR analysis. Primer sequences for all DNA fragment markers, ESTs and genes can be found in GDB (URL <http://gdbwww.gdb.org/>) or by referring to Bouzyk et al. [21]. Primers 5'TACACCAGGATCAAG-AAGGC3' and 5'AGAGAAACCGAGAAGAAACC3' for RD55 were designed by Vivienne Watson [pers. commun.]. Oligonucleotides for TYRP1 and for CNTFR [21] are also available. Additionally, primers 5'TTGATGACCTGACTGGGGAGC3' and 5'GGC-TTTGTTTGGTCTTCATAAGCC3' for AK3 were obtained from Steve Jeremiah [pers. commun.]. All PCR products were sized separated by electrophoresis on 2% agarose gels and visualized with 0.5 µg/ml ethidium bromide. Loci were scored as present, absent or ambiguous (present in only one PCR reaction) for each RH hybrid and as present or absent for each YAC.

### *YAC Screening and Construction of YAC Map*

YACs spanning the short arm of chromosome 9 were chosen from the CEPH mega-YAC library [28]. By scanning the library using the Quickmap database navigation tool, we identified and pooled 130 partially localized YACs for STS content mapping, starting initially with RH framework markers. Pooling YACs on the basis of coarse localizations made it possible to quickly 'bin' new markers relative to the RH framework markers. A cocktail of YACs chosen from levels 1, 3 and 5 was sufficient to give about 90% coverage of the short arm of chromosome 9. The 130 YACs screened were placed in 13 DNA primary pools representing 10 clones each. After PCR of primary

pools, positive YAC addresses were confirmed in secondary pools. Tiling contigs of YACs could be constructed based on PCR hits against respective markers.

#### *RH Statistical Analyses*

The Anneal program [Barrett J., pers. commun.], used a simulated annealing method to find an optimal order by minimizing the number of obligatory breaks. Maxreal, part of the same package, used maximum likelihood methods to find the best order by looking at all orders of a specified subset of probes. Retention patterns were examined, the equiprobable retention model was used and the map enlarged with the RHMAP software package version 2.01 [29]. Additional probes were added to a framework map [21] by 'stepwise locus ordering', and the order was confirmed by maximum likelihood methods. The process of adding loci continued until no additional loci could be uniquely positioned with the required support of the 1,000:1 likelihood ratio. The final framework map was verified using RHMAP. Markers which could not be uniquely positioned on the framework map with the required level of support were assigned to a range of (usually adjacent) intervals. Distances between loci were calculated using the function  $D = -\ln(1-o)$ , where  $D$  is the distance in rays (R) and  $o$  is the frequency of breakage between two markers for a given set of loci [22].

#### *Fluorescence in situ Hybridization*

FISH and reverse painting [30, 31] (where, in this case, DNA from a YAC was used as a probe to 'paint' normal human chromosomes) were used to assign chromosomal regions to specific YACs. Chromosome metaphase spreads were prepared using standard cytogenetic techniques. Nick translation using a commercially available kit (BRL) was used to produce biotinylated *Alu*-PCR products from individual hybrids. Biotinylated probe DNAs were separated from unincorporated nucleotides using Sephadex G-50 columns. The labeled probe was ethanol precipitated and resuspended in a standard hybridization mix containing 50% formamide, 20% dextran sulfate and  $2 \times$  SSC (pH 7.0). After denaturation and preannealing, probes were hybridized to metaphase chromosomes and detected as described [32].

#### *Construction of an Integrated Map*

Markers positioned unambiguously on the YAC map were integrated into the RH framework map. A number of the unambiguous markers on the YAC map were only approximately positioned on the RH map. Both the RH framework markers and unambiguous markers on the YAC map formed an 'integrated' framework used to assign other markers to produce a map containing 65 loci. All markers were placed in their most likely position, based on this strategy and on results from previous unpublished data [M.B.]. The integrated map does not conflict with the individual RH or YAC maps (fig. 4).

## **Results**

#### *RH PCR and Statistical Analysis*

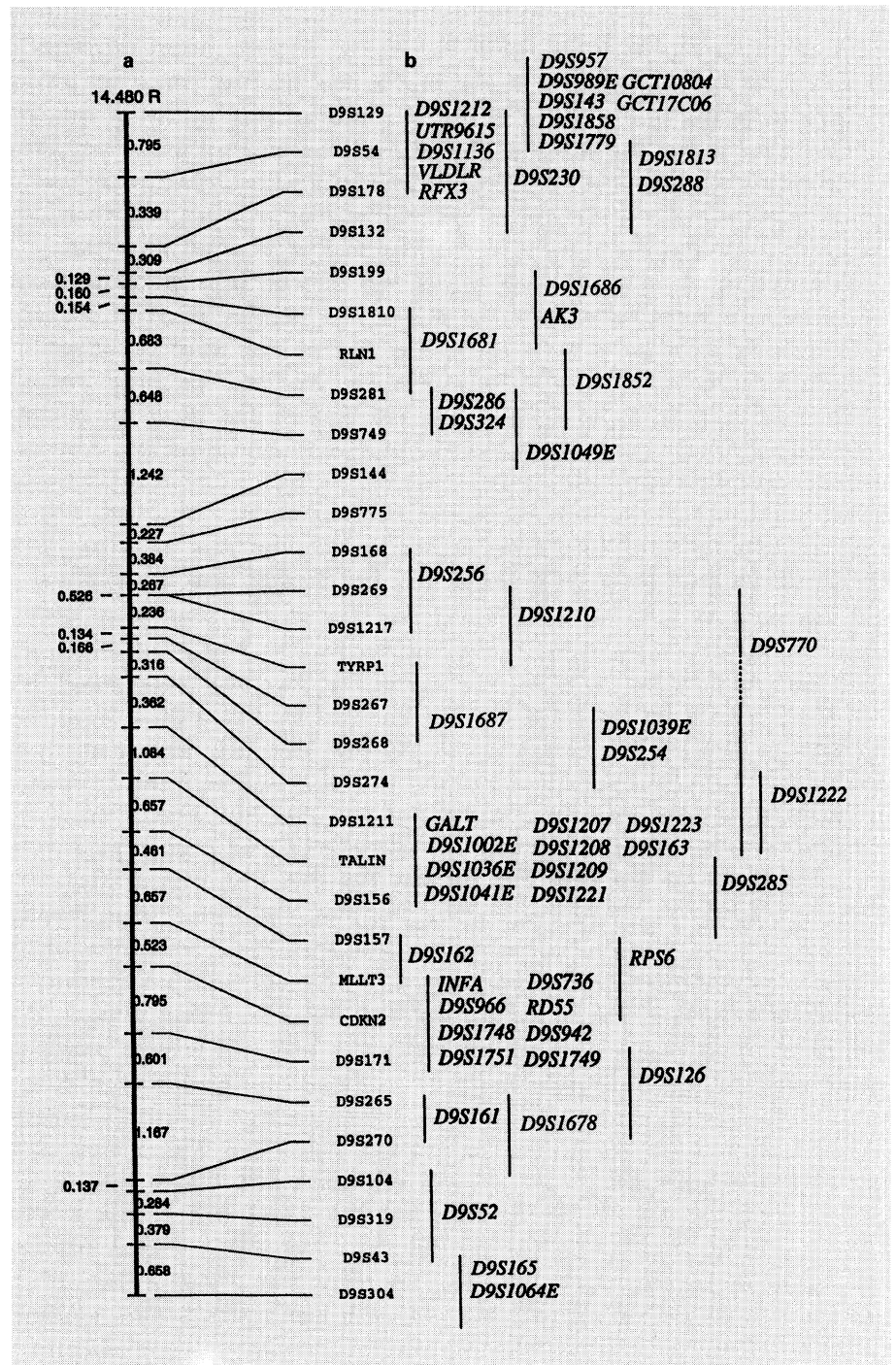
Sixty-four markers were screened by PCR against a panel of 93 *Alu*-positive clones including 2 positive (chromosome-9-specific genomic DNA and total human genomic DNA) controls and 1 negative (hamster) control in

order to build on the 9p RH map previously constructed using 50 other markers with the same panel [21]. There were sufficient stocks of DNA for each independent RH clone in the panel from this previous study in order to type all 64 new markers, and no RH clone was regrown. The number of products generated for all 114 markers for each hybrid ranged from 1 to 90. Only 1 hybrid from the panel did not give any products with the 114 markers.

PCR data has been scored in duplicate for the presence or absence of single-copy DNA. Of all individually typed reactions, 0.3% were ambiguous (positive in one run, ambiguous in the other run) and were treated as positive in all analyses. The average retention frequency for all 114 markers was 23%, with retention frequencies ranging from 8 to 31%. Of these 114 markers analyzed, 13 were STSs for genes and 10 were ESTs.

Statistical analysis outlined earlier has been used to extrapolate a map from these data. A total of 31 markers were ordered, with odds of support greater than 1,000:1 (fig. 1). Fifty-six other markers which could not be placed in a single interval with 1,000:1 support have approximately been placed on the framework map next to a bar showing the range of intervals which fall within the 3-lod unit support interval. A dashed line joins regions of high support, but is itself not supported. The map spans a distance of 1,448.0 cR and distance estimates between adjacent loci are shown (fig. 1).

This map includes 2 potential tumor suppressor genes, 1 coding for the cytoskeletal protein talin which has been assigned between markers D9S1211 and D9S156, and CDKN2 which has been positioned 79.5 cR telomeric to D9S171, both placements supported with odds greater than 1,000:1. RLN1 has been placed on 9p24 between D9S1810 and D9S281, with odds greater than 1,000:1. The genes TYRP1 and MLLT3 have also been placed on the framework map. RPS6, INFA, GALT, AK3, VLDLR, RFX and 7 ESTs could not be placed in a single interval and have been placed within a range of intervals which fall within the 1,000:1 support intervals. Interestingly, 3 of the 7 ESTs (D9S1002E, D9S1036E and D9S1041E) cluster around the TALIN locus. CNTFR, ALDH5, D9S1021E, D9S1023E and D9S1027E could not be placed with high levels of support to any specific region on the short arm. Similarly, 22 other markers contained multiple intervals not adjacent to each other with 1,000:1 support and so are not included on the map. The RH framework agrees well with other maps currently published including those by EUROGEN [14] and Généthon [15, 16].



**Fig. 1.** RH map of chromosome 9p. **a** A total of 31 markers were ordered on a framework map, with odds of support greater than 1,000:1. **b** Fifty-six other markers which could not be placed in a single interval with 1,000:1 support have approximately been placed on the framework map next to a bar showing the range of intervals with 1,000:1 support.

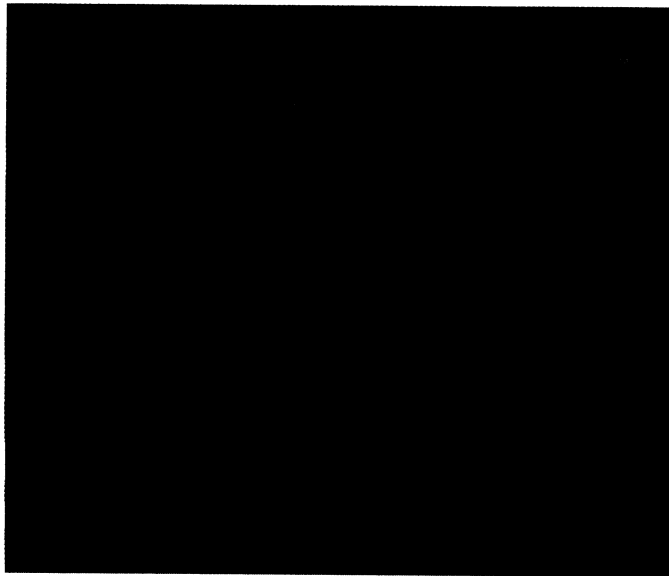
### Cytogenetic Analysis

Twenty YACs were selected to ascertain cytogenetic location by FISH. This analysis was to confirm chromosomal content and determine whether YACs retained specific regions as defined by relevant markers. DNA from YAC 804-b-9 (fig. 2) was labeled by *Alu* IV PCR produc-

tion [33] and then reverse painted by hybridization to a metaphase spread, resulting in localization to 9p22. These results provide support for the broad correlation of PCR data with markers on the YACs and their localization by FISH to a corresponding chromosomal region.

*YAC PCR Analysis*

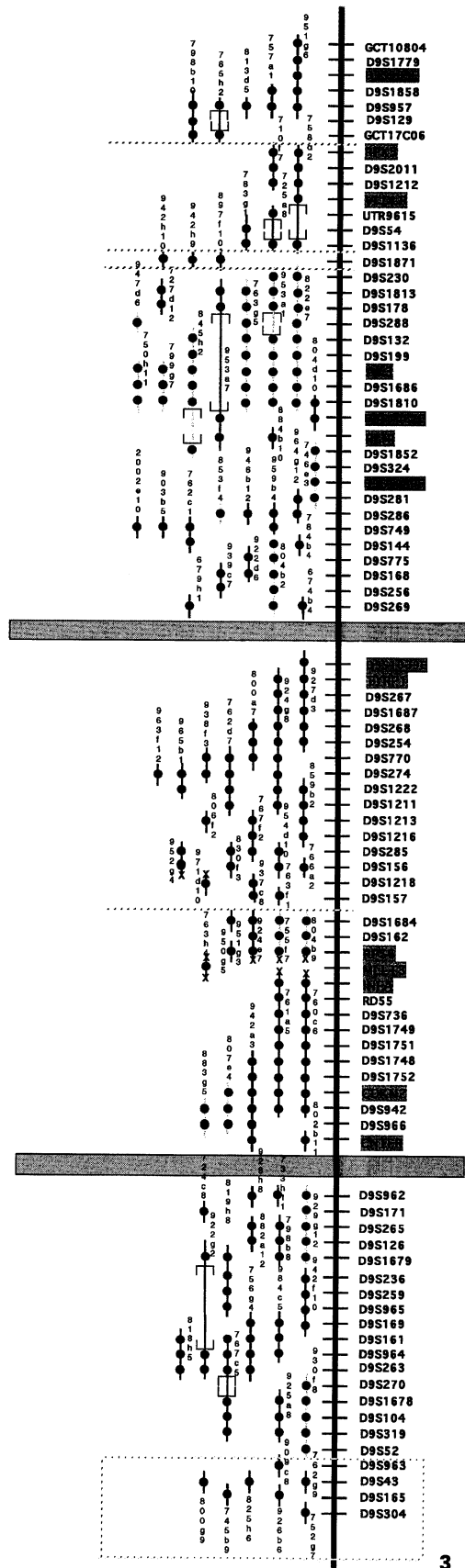
Eighty-nine of the 114 markers typed by PCR against the RH panel produced at least 1 YAC address (fig. 3). Eighty-eight individual YACs (average size 0.9 Mb) formed a series of contigs against these markers. Ten genes and 4 ESTs (including CNTFR and D9S1027E which could not be placed on the RH map) were included in the YAC map. Additionally, there were a total of 16 other markers on the YAC map not placed on the RH map. 4 minor gaps (<1 Mb), 2 large gaps of about 2–3 Mb and a poorly defined region near the centromere between D9S52 and D9S304 (~1–2 Mb) suggest ~90% coverage of the short arm. Thirty-five markers out of 89 could not be separated.



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**Fig. 2.** Cytogenetic analysis of YAC 804–b–9. The chromosomal content of YAC 804–b–9 was determined by FISH onto a metaphase spread of normal blood in order to confirm the YAC location and determine the presence or absence of chimerism. This result provides support for the broad correlation of PCR data with markers on this YAC and its localization by FISH to a corresponding chromosomal region (9p22).

**Fig. 3.** YAC map of chromosome 9p. All markers on the YAC map have been screened on the RH panel. The minimum tiling path on contigs represents approximately 90% coverage of the short arm. There are 2 major gaps averaging 2–3 Mb each on the map (yellow bars). A red dashed line indicates a minor gap. A green X indicates an *Alu*-PCR/fingerprint relationship between 2 YACs or a level 2 or higher relationship. Ten gene STSs (green highlights) and 4 ESTs (pink highlights) are included. Other YACs which have been assigned by FISH are indicated by vertical yellow lines. An open bracket on a YAC indicates a negative PCR for a particular marker, and a blue dot indicates a positive PCR for a particular marker. There are no conflicts with the RH map.



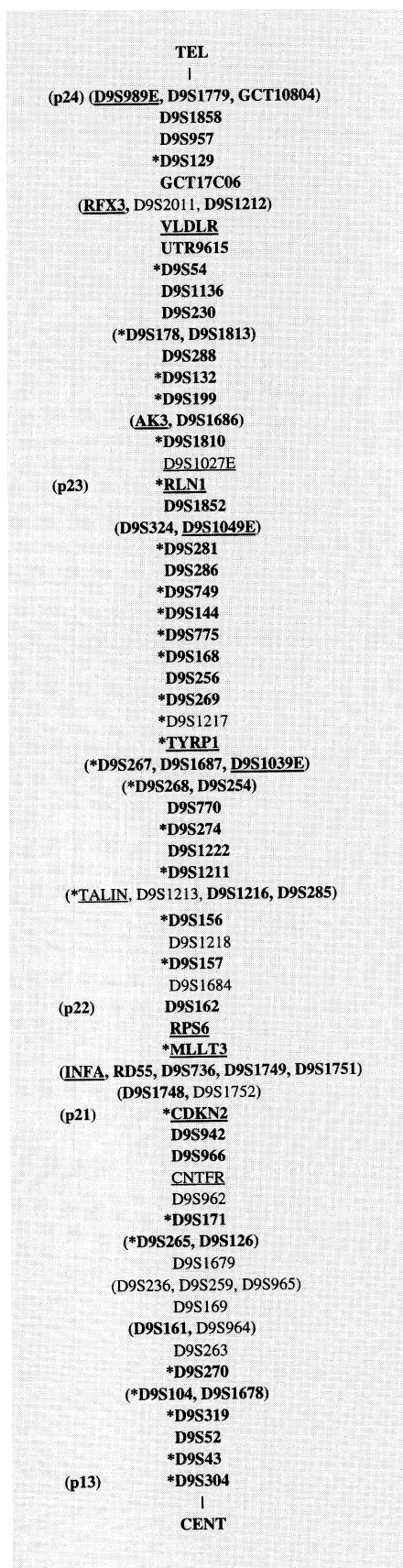
3

## Discussion

A RH map composed of 87 markers assigned to human chromosome 9p was produced with a 6,000-rad radiation-reduced hybrid panel from an original complement of 114 markers. Marker retention frequencies ranged from 8 to 31%, averaging 23%, suggesting that nearly a quarter of the chromosome may have been retained per hybrid (roughly averaging 26 positive PCR products per hybrid for the 114 markers). However, an indication of the different relative amounts of chromosome 9p retained per hybrid can be seen from the considerable variation in the range of marker-specific products generated by PCR, from 1 to 90 out of the 114 markers typed.

The retention data was analyzed using multipoint methods which rely upon maximum likelihood. The order was determined with the model allowing for equal retention probabilities for all markers, and independent fragment retention was also assumed for each individual RH. An alternative marker retention model could be used as there is evidence from various sources suggesting that fragments near the centromere may be retained at higher frequencies [25, 34–36]. Retention frequencies of 114 markers ordered on the framework map show no wide variation, and there is no evidence of a 'centromeric' effect. Of the 27 markers which could not be placed on the RH map, the retention frequency was ~20% which suggests that poorly placed markers are not dependent on retention frequencies.

The physical length of chromosome 9p has been estimated to be 51 Mb [37]. This is based on an estimate of the relative length of the genome contained in chromosome 9p, given an estimate of the total genomic length in megabases. The total length of our RH map was found to be 1,448.0 cR. This leads to an estimate of about 35 kb per  $cR_{6,000}$  for the framework map. The average resolution of the RH framework map is 48.3  $cR_{6,000}$ , which approximates to a physical distance resolution of 1.7 Mb between uniquely placed markers. The smallest interval on the framework map is 12.9  $cR_{6,000}$ , which has an estimated



**Fig. 4.** Integrated map of chromosome 9p. The map contains 65 loci including 51 uniquely placed markers with an average resolution of 0.79 Mb. RH framework placements in conjunction with YAC information have been used to create a RH/YAC map. Markers with an asterisk indicate RH framework markers. Markers in bold indicate common markers to both RH and YAC maps. Eleven genes and 4 ESTs (underlined) are included in the map. Broad cytogenetic locations are indicated. TEL = Telomere; CENT = centromere.

physical distance of 454 kb, the largest interval being 124.2 cR<sub>6,000</sub> (4.37 Mb). When comparing YAC distance estimates with cR<sub>6,000</sub> distance estimates, the values are fairly consistent when such estimates can be made and considering other possible problems such as YAC chimerisms/deletion/rearrangements. For example, between D9S268 and D9S1211, the distance is 67.8 cR<sub>6,000</sub> (2.37 Mb). The YAC tiling maximum distance for this region is 3.08 Mb. If one does not allow for any YAC overlap, the distance covered by the YAC contig for this region is only 1.3 times greater than the RH map distance estimates. Where it was feasible, these RH/YAC distance estimate comparisons were carried out on other regions of 9p. In no case does the projected YAC physical distance increase over the RH distance estimate by more than twofold (results not shown), always remaining between one and twofold. Moreover, analysis of different chromosomes provided evidence that RH map distances are not proportional to the physical distance [22, 38–41]. In addition, analysis of smaller subregions of the same chromosome can lead to variations in the kb/cR relationship [39].

Of the 87 markers included in the RH map, 69 have a YAC address. Intriguingly, 11 of the 16 markers which do not have a YAC address cluster around the TALIN locus, including 3 ESTs and GALT as well as TALIN itself. One of these ESTs, D9S1002E, has 100% homology to a gene coding for tropomyosin, suggesting a possible clustering of genes coding for cytoskeletal proteins to this region.

The RH map was able to order two sets of markers at odds greater than 1,000:1 which could not be uniquely placed on the Généthon genetic map; D9S267/D9S268 and D9S171/D9S265. Furthermore, YAC information could separate other nonuniquely assigned sets of markers from the Généthon map (D9S1779/D9S1858, D9S1871/D9S1813, D9S178/D9S288, D9S1686/D9S1810, D9S256/D9S269, D9S274/D9S285, D9S1684/D9S162, D9S171/D9S265/D9S1679, D9S161/D9S270/D9S1678).

The CEPH mega-YAC library has produced sufficiently large numbers of YACs for high-redundancy coverage of most of the human genome after exhaustive screening with PCR-based probes, *Alu*-PCR probes and fingerprinting [28]. Our analysis has shown that the CEPH mega-YAC library is very representative of 9p as tiling paths have been established over most regions, and 67% of YACs analyzed by us are localized to this part of the chromosome. Additionally, cytogenetic data from FISH analysis of 20 YACs has found 11 localized to 9p only, 5 with signals on 9p and other chromosomes and 4 with no signals. Thus, the PCR data complements the FISH analysis, although there is some degree of chimerism.

The YAC map (fig. 3) contains a number of minor gaps (marked by red dashed lines) which are insignificant based on current physical evidence in the respective regions. These gaps should be able to be closed using methods such as *Alu*-PCR or hybridizations with neighboring YACs. The region from D9S963 to D9S304 is poorly defined, probably due to its proximity to the centromere. There are 2 major gaps (fig. 3, yellow bars). Mapping evidence suggests that these gaps average 2–3 Mb each. The minor gaps next to RFX3 and D9S1871 and both major gaps next to D9S269 and D9S962 also occur in the Whitehead STS-YAC map [19]. Additionally, the Whitehead map has gaps between D9S281/D9S144 and D9S156/D9S157 which are closed on our YAC map. Furthermore, there is no information in the CEPH YAC contig map [18] for the gaps at RFX3 and D9S1871 as the contigs only begin to be assembled centromeric to D9S288. Similarly, the CEPH map has a gap next to D9S269, and the region around D9S962 is poorly defined. Our YAC map thus supplements the information provided by both Whitehead and CEPH YAC maps.

Whole-genome-mapping efforts occasionally have to be viewed with caution. For example, the Whitehead STS map places D9S163 as its most telomeric marker. This marker is placed around the TALIN locus on our RH map, and other groups have also found major conflicts with this marker and the Whitehead map [Rebello M., and Guioli S., pers. commun.]. Moreover, the Whitehead STS map contains less than half of the markers placed on our maps. Of the 13 genes localized on our maps, 7 are not included in the recently published gene map of the human genome [20]. These are AK3, RLN1, RPS6, MLLT3, CDKN2, CNTFR and TALIN. The 6 genes which are included make up a total complement of 47 uniquely identified transcripts on the Schuler map in this region. Therefore, our RH and YAC maps provide complementary and refined information in addition to the information provided by whole-genome-mapping efforts.

There is only one conflict between our YAC map and the Généthon genetic map [16] where, on our map, D9S263 is placed telomeric to D9S270/D9S1678. The opposite is the case with the Généthon map. However, the Généthon map places D9S263 and D9S270/D9S1678 1 cM apart, with low odds of placement (less than 1,000:1).

We have taken the low-resolution RH framework map as a starting point for conversion into a high-resolution RH/YAC integrated map (fig. 4). From the 114 markers screened, 71 were common to both RH and YAC maps, and 70 are included in the integrated map. Only

9 markers (D9S50, D9S55, D9S200, D9S1948, D9S970, D9S1224, D9S1021E, D9S1023E, ALDH5) could not be placed on either map.

Many of the microsatellites which have not been ordered on the Généthon map could be ordered here, in many cases with a higher resolution than in previous maps. The broad agreement of our integrated map and other reports suggests that our RH panel and YAC pools will be valuable for mapping markers and as a cloning resource for this chromosomal region. This integration of additional microsatellite markers, other STSs and genes on our RH/YAC map should provide a useful starting point for positional cloning of disease genes such as GLDC, VMCM, the constitutional 9p syndrome and sex determination gene and potential tumor suppressor genes such as the CDKN2 locus and TALIN.

Finally, using our dual approach of the exploitation of RH mapping technology with CEPH data and the mega-YAC library should not only greatly aid the completion of a high-resolution map of the human genome, but also provide the platform for the generation of sequence-ready regions of the whole of the short arm of chromosome 9.

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