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Localization of a putative tumor suppressor gene by using homozygous deletions in melanomas

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ABSTRACT **The p21 region of human chromosome 9 is thought to contain a gene (*MLM*) involved in genetic susceptibility to melanoma and a gene or genes that influence progression of certain other tumors. Genomic clones that span a large region in 9p21 surrounding the presumptive tumor suppressor gene(s) have been isolated. A set of sequence-tagged sites in this region has been developed. By using these markers and others previously reported, the 9p21 region has been studied by physical mapping in 84 melanoma cell lines. A putative tumor suppressor gene, perhaps *MLM* itself, has been localized to a region of less than 40 kb that lies proximal (centromeric) to the α-interferon gene cluster.**

The incidence of melanoma is increasing at a rate second only to the rate of lung cancer in females. Approximately 32,000 new cases of cutaneous malignant melanoma were diagnosed in the United States in 1992, and although the disease is readily treatable in its early stages, it causes 7000 deaths in the U.S. per year at present (1). A considerable portion of the incidence of melanoma can be attributed to environmental factors such as exposure to sunlight (2). However, extensive studies of melanoma-prone families have shown that susceptibility to melanoma is controlled in part by a locus known as *MLM* on the short arm of chromosome 9 in humans (3–5). Melanoma predisposition in these families is inherited as a dominant Mendelian trait closely linked to the α-interferon gene family.

MLM or a closely linked locus may be involved in the genesis of tumors other than melanoma. Cytogenetic and/or molecular abnormalities in the vicinity of *MLM* have been characterized in glioma cell lines (6), non-small cell lung lines (7, 8), and acute lymphoblastic leukemia lines (9, 10). These abnormalities include chromosomal translocations, inversions, heterozygous deletions, and homozygous deletions. Whether or not *MLM* contributes to heritable predisposition to cancers other than melanoma is unclear. However, based on the frequency of 9p21 chromosomal abnormalities in nonmelanoma tumor cells, it is probable that the *MLM* region contains a gene (or genes) that participates at least in the progression of several different tumor types.

To characterize the *MLM* region further, we studied a large set of genetic markers and sequence-tagged sites (STSs). These studies enabled us to (*i*) develop a physical map of the region, (*ii*) determine the boundaries of the minimal area deleted frequently in melanoma cell lines, and (*iii*) determine within the limits of genetic resolution that *MLM* lies in the same region as the area defined by homozygous deletions.

MATERIALS AND METHODS

Tumor Cell Lines. Seventy six melanoma cell lines were obtained from the Ludwig Institute for Cancer Research, Memorial Sloan–Kettering Cancer Center, and eight melanoma cell lines and five nonmelanoma lines were from the American Type Culture Collection (ATCC).

Preparation and Analysis of Tumor Cell Line DNA. DNA was isolated from cell lines by the addition of 1–2 × 10^6 cells to 3 ml of lysis buffer (0.1 M NaCl/0.1 M Tris·HCl, pH 8.0/5 mM EDTA/0.5% SDS), followed by vortex mixing and incubation at 65°C for 30 min. A 0.5-ml aliquot of 8 M KOAc was added, and the reaction mixture was mixed and incubated on ice for 30 min. After centrifugation (5 min at 10,000 × *g*), the supernatant was precipitated with an equal volume of 95% ethanol and centrifuged again (15 min at 10,000 × *g*). The DNA was resuspended in 50–200 μl of H_2O.

For PCRs, 50 ng of template was added to 30 pmol of each oligonucleotide primer in a 20-μl reaction mixture that contained all four dNTPs (each at 0.1 mM), 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, and 1 unit of AmpliTaq polymerase (Perkin–Elmer). Samples were cycled in a Perkin–Elmer 9600 thermal cycler 35 times at 94°C for 10 sec, 55°C for 10 sec, and 72°C for 10 sec. The products were visualized after electrophoresis through either 1.5% agarose (SeaKem, FMC) or 3% NuSieve 3:1 agarose (FMC) by ethidium bromide staining.

YAC, P1, Cosmid, and λ Genomic Clones. Yeast artificial chromosomes (YACs) containing markers in the *MLM* region were obtained by screening the Centre d'Etude Polymorphisme Humain (CEPH) YAC libraries with *IFNA*, *D9S171*, and *D9S126* by using PCR conditions described above. Yeast strains containing YACs were grown at 30°C for 3 days with vigorous shaking in AHC medium [casein hydrolysate-acid (10 g/liter)/yeast nitrogen base (1.7 g/liter)/ammonium sulfate (5 g/liter)/adenine hemisulfate (20 mg/liter)/2% (wt/vol) glucose, pH 5.8]. Yeast DNA was prepared as described (11).

For phage library construction, yeast genomic DNA containing YAC DNA was digested to completion with *Bam*HI, inserted into *Bam*HI-digested EMBL3 phage arms (Promega) by using T4 DNA ligase (Boehringer Mannheim), and packaged *in vitro* with Gigapack II extracts (Stratagene). Phage were grown on *Escherichia coli* C600. Recombinant phage containing human DNA were identified by hybridization with ^{32}P-labeled human Cot-1 DNA (GIBCO/BRL). Phage including human sequences joined to YAC vector (end clones) were identified by screening with PCR fragments containing sequences from the YAC left or right arm. Hybridization and washing were carried out under standard conditions (10). Positive plaques were picked and purified by replating three times. Phage DNA was prepared using Qiaex columns (Qiagen, Chatsworth, CA).

For cosmid library construction, yeast genomic DNA containing YAC DNA was digested partially with *Sau*3A and

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Abbreviations: STS, sequence-tagged site; YAC, yeast artificial chromosome; CEPH, Centre d'Etude Polymorphisme Humain.

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fractionated by size on a linear (10–40%) sucrose gradient as described in Maniatis *et al.* (12). SuperCos 1 cosmid vector (Stratagene) was prepared according to manufacturer's directions, mixed with insert DNA at a mass ratio of 4:1 (insert/vector), treated with ligase, and packaged *in vitro* as described above. Cosmids were introduced into DH5α host cells and plated at a density of 2000 colonies per 15-cm Petri dish. Colony hybridization was carried out as described above and in Maniatis *et al.* (12).

P1 clones spanning the *MLM* region were obtained from Genome Systems, St. Louis, by screening with STSs that we provided. DNA from these clones was isolated by alkaline lysis (13) followed by cesium chloride gradient centrifugation (12).

Generation of STSs. STSs were generated by sequencing 1.0 μg of P1, cosmid, or λ template DNA with oligonucleotides complementary to sequences flanking the cloning site of the P1 vector (pSacBII) SuperCos 1 vector, or the EMBL3 vector. Sequencing was done on an ABI model 373A DNA sequencing system with the PRISM Ready Reaction Dye-Deoxy Terminator Cycle sequencing kit (Applied Biosystems). STSs were designed to be as close as possible to 20 bp long and to have a melting temperature as close as possible to 60°C.

RESULTS

Localization of *MLM* by Using Genetically Linked Markers. To analyze tumor cell lines for homozygous deletions in the 9p21 region, we initially used a set of markers known to be linked to *MLM*. These markers were used originally to demonstrate dramatic linkage (logarithm of odds score = 12.7) of melanoma predisposition in 10 Utah kindreds and 1 Texas kindred (3). The markers included a sequence from the α-interferon gene cluster (*IFNA*) (14), which was the most distal marker tested, a proximal marker (*D9S104*), and four additional markers in between (*D9S171*, *D9S126*, *D9S161*, and *D9S169*) (3). From genetic studies, the linear sequence of the intervening markers was thought to be *D9S171*, *D9S126*, *D9S161*, and *D9S169*. The *IFNA* marker consisted of an oligonucleotide primer pair that amplified two fragments from wild-type genomic DNA: a roughly 138- to 150-bp polymorphic fragment (IFNA-l) that contains a poly(CA) stretch and a roughly 120-bp invariant fragment (IFNA-s). The location of IFNA-s with respect to IFNA-l was unknown. Because the α-interferon genes form a family of linked genes, it was possible that IFNA-s was within this gene cluster.

Five nonmelanoma tumor cell lines reported previously to contain deletions were analyzed using the set of genetic markers. Each cell line revealed homozygous deletions of at least one of the markers tested (Table 1). No homozygous deletions were identified using *D9S161*, *D9S169*, or *D9S104*. The minimum region of overlap among these deletions was flanked by IFNA-l and *D9S171*. This suggested that the region between these two markers contains a gene(s) that is involved in tumor suppression, possibly *MLM*. We therefore focused attention on the genomic region between *D9S171* and IFNA-l, particularly in the vicinity of IFNA-s.

Genomic Clones in the *MLM* Region. To obtain genomic clones of the region surrounding IFNA-s, CEPH YAC libraries were screened (15). Six YACs were identified that contained the *D9S171* marker and five were identified that contained IFNA-s. No YACs were isolated that included both *D9S171* and IFNA-s (Fig. 1). Three of the YAC clones (C9, C6, and F9) were subcloned into λ phage and one YAC (C6) was subcloned into a cosmid vector. These λ and cosmid clones provided a convenient way to produce STSs internal to known genetic markers and to expedite the chromosomal walk described below.

To provide an independent source of genomic DNA for construction of a contiguous genomic map of the region and to aid in production of STSs, we initiated a chromosomal walk in P1 clones from IFNA-s extending toward *D9S171*, from *D9S171* extending back toward IFNA-s, and from the two ends of YAC C6, a nonchimeric YAC, in both directions. A total of 27 P1 clones were isolated as part of this chromosomal walk (Fig. 1). The ordered Pls formed a contiguous assembly that stretched from IFNA-s to *D9S171* with two gaps. P1 clones and several phage and cosmid clones were used to generate a fine structure map of the *MLM* region.

Fine Structure Analysis of *MLM* Region. To construct a more detailed molecular map of the *MLM* region, additional markers were required. DNA sequences obtained from the genomic clones were used to design PCR primers for STSs. These STSs served in turn to help order the P1 and YAC clones. A total of 54 STSs from the region between IFNA-s and *D9S171* were the primary basis for developing a detailed physical map of the *MLM* region (Fig. 1). These STS primer sequences can be obtained from the authors and their approximate positions on the physical map shown in Fig. 1 can be determined by their P1 names.

This set of markers stretching from IFNA-s to *D9S171* was used to test 84 melanoma cell lines for homozygous deletions in the *MLM* region. A total of 47 lines revealed regions of homozygous deletion (Fig. 2). Several of the deletions were extensive; for example, nine lines were missing a region that included both 816.7 and 760-L.

For the purpose of localizing *MLM*, the most informative tumor lines fell into two groups (Fig. 2): (*i*) those that contained deletions of c5.1 alone (class 11) and (*ii*) those that contained deletions of c5.3 alone (class 12). A total of six melanoma lines fell into these categories. In all cases where deletions were detected, the deletion appeared to be simple; that is, there was no evidence of multiple deletion events in the region between IFNA-s and *D9S171*. Thus the lines harboring deletions delineated a region of deletion overlap centered around markers c5.1 and c5.3, making the devel-

Table 1. Homozygous loss of chromosome 9p markers

	Markers						
Tumor cell line	IFNA-1	IFNA-s	*D9S171*	*D9S126*	*D9S161*	*D9S169*	*D9S104*
U-138	−	−	−		+	+	+
U-118	−	−	−	−	+	+	+
U-87	−	−	+	+	+	+	+
A-172	+	−	+	+	+	+	+
H4	−	−	−	−	+	+	+
SK-Mel-5	+	−	−	−	+	+	+
SK-Mel-144	−	−	−	+	ND	ND	ND

Homozygous deletions in tumor cell lines detected with genetic markers linked to *MLM* in previous studies (3). IFNA-1 is distal and *D9S104* is proximal. U-138, U-118, U-87, A-172, and H4 are neuroblastomas or gliomas from the ATCC. SK-Mel-5 and SK-Mel-144 are melanoma lines from the Sloan-Kettering collection. ND, not done; +, detected; −, not detected.

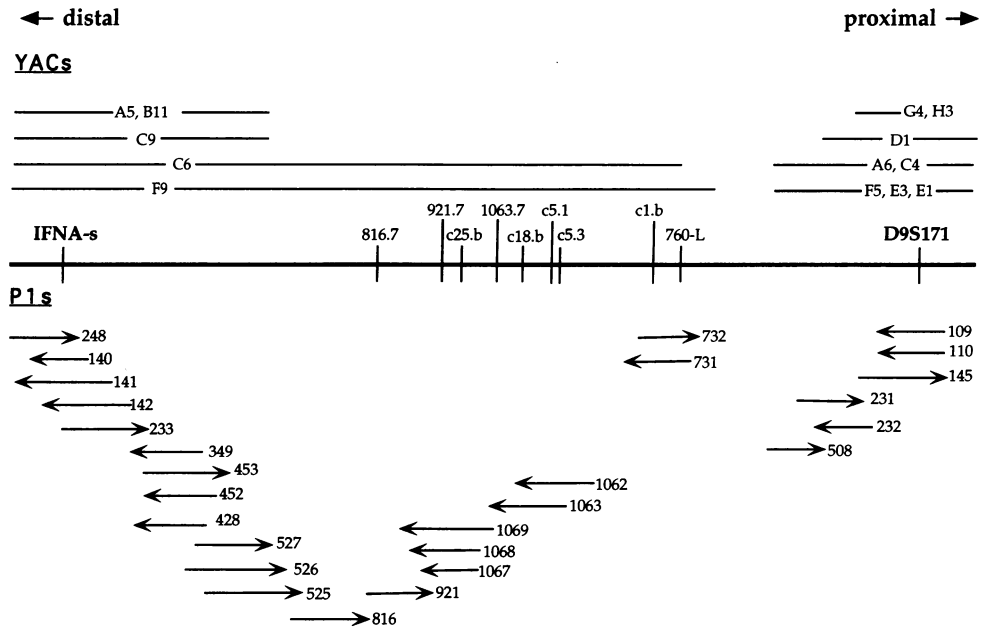

FIG. 1. YAC and P1 clones in the region bounded by IFNA-s and *D9S171*. The centromere is to the right. For P1 clones, the arrow points in the direction of the T7 promotor sequence in the vector and the numbers permit identification of specific P1s. YACs that are grouped together represent clones that are similar based on mapping STSs in the region. These YACs are presumed not to be identical. YACs A5, B11, C6, C9, and F9 contain IFNA-1 and IFNA-s. YACs D1, F5, and E3 contain *D9S126* and *D9S171*. Neither the proximal ends of YACs that include *D9S171* nor the distal ends of YACs that include IFNA-s are shown. Distances are not necessarily drawn to scale. The markers internal to IFNA-s and *D9S171* are depicted in Fig. 2. Markers that begin with "c" are derived from cosmid end sequences. The cosmids are not shown. The distances between c1.b and c5.3 and between 760-L and *D9S171* are unknown. YAC names are as follows: A5, 761A5; B11, 802B11; C9, 887C9; C6, 760C6; F9, 886F9; G4, 942H5; H3, 943B12; D1, 465E12; A6, 326C10; C4, 367H10; F5, 798B8; E1, 181G2.

opment of a complete physical map of the region from IFNA-s to *D9S171* unnecessary.

DISCUSSION

Placement of Genetic Markers. Analysis of YAC clones and of deletions in tumor lines yielded results consistent with the genetic placement of markers: *IFNA*, *D9S171*, and *D9S126*. Two YACs contained both *D9S171* and *D9S126* and four YACs contained IFNA-l and IFNA-s (Fig. 1). None contained both *D9S171* and *IFNA*. These data suggested (*i*) that IFNA-l and IFNA-s are closely linked and (*ii*) that *D9S126* and *D9S171* are linked. These results were confirmed by cell line deletions. Most cell lines that were missing *D9S171* also lacked *D9S126*. Conversely, line U-87, although testing positive for *D9S171* and *D9S126*, lacked IFNA-s and IFNA-l

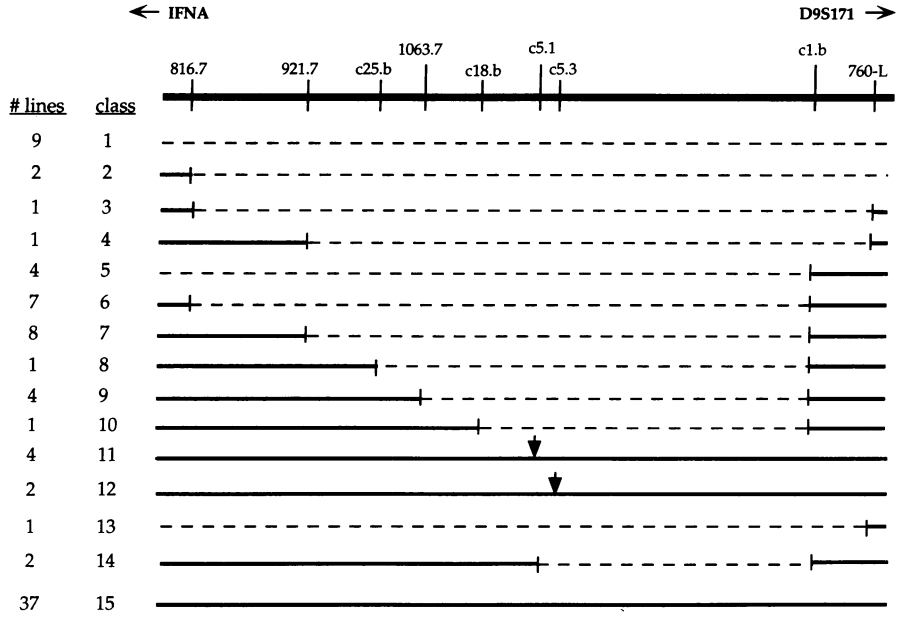

FIG. 2. Diagram of deletions, indicated by dashed lines, observed in melanoma cell lines. The cell lines fell into 15 classes based on the set of markers deleted. The number of representatives of each of the 15 classes is shown in the column labeled "# lines." The locations of the deletion breakpoints for classes 2–10, 13, and 14 are portrayed as falling at the marker adjacent to the deleted DNA; that is, the last positive marker in the series leading up to the deletion. For classes 11 and 12, the sites of deletions are shown by vertical arrows because the deletions remove a single marker. A list of all cell lines used in this study is available by request to the authors.

(Table 1). One melanoma cell line, SK-Mel-5, lacked IFNA-s, *D9S171*, and *D9S126* but not IFNA-l. Thus, IFNA-l must be distal to IFNA-s. Another melanoma line, SK-Mel-144, contained a deletion that included IFNA-l, IFNA-s, and *D9S171* but not *D9S126*, placing *D9S171* between IFNA-s and *D9S126*. Collectively, these findings are consistent with the marker order given in Table 1.

Physical Distance Between Genetic Markers. Our results did not permit a precise estimate of the distance between IFNA-s and *D9S171* as we were unable to isolate YACs that contained both markers. Furthermore, based on mapping with STSs, none of the 5 YACs that extended distal from IFNA-s overlapped any of the 12 YACs that extended proximal from *D9S171*. Given that CEPH YAC inserts average less than 500 kb in length, the distance between IFNA-s and *D9S171* is likely to be at least this large.

As discussed below, c5.1 and c5.3 are especially interesting markers based on their involvement in melanoma deletions. They were derived from a cosmid (c5) and are, therefore, within one cosmid length of each other. The region between IFNA-s and c5.3 was covered by nine walking steps in a P1 library. By assuming that each step is on average the length of half a P1 insert, the distance between c5.3 and IFNA-s is roughly 400 kb.

Deletions in Tumor Lines. Homozygous deletions of the 9p21 region were found in 56% of melanoma tumors tested. Eleven tumor lines contained deletions that extended on the proximal side through 760-L and 14 lines contained deletions that stretched beyond 816.7 on the distal side. By assuming the deletions of gene(s) in this region contribute to the tumor phenotype, the tumor suppressor gene(s) must also lie between 760-L and 816.7. The smallest deletions involved markers c5.1 and c5.3. Of all the markers tested, c5.3 was deleted from the largest number of lines, 43 lines. Therefore, the most probable position of the tumor suppressor gene(s) is very close to c5.3. Two lines contained deletions of c5.3 alone (class 12) and four lines lacked c5.1 alone (class 11). Both these markers were present on the c5 cosmid. Thus, it is likely that the tumor suppressor gene(s) includes sequences from cosmid c5.

The results presented here are consistent with previous genetic studies of *MLM* (3) that found the region between IFNA-1 and *D9S126* to be the most probable location for *MLM*. Recent genetic studies have confined the location of *MLM* further using a polymorphic (CA) repeat that lies between IFNA-s and C5.3 on P1-452 (Fig. 1). Analysis of a recombinant chromosome using this marker places *MLM* proximal to P1-453 (16). Thus, *MLM* maps within the region where homozygous deletions in melanoma cell lines cluster.

These results support the view that the *MLM* tumor suppressor locus is located somewhere near c5.3. All the lines that contained deletions shared a common area of deleted DNA, with the exception of the set whose deletions were restricted to c5.1 or c5.3 (classes 11 and 12). There was no indication of nonoverlapping deletions in this panel of cell lines other than those within cosmid c5. Therefore, there is no basis to invoke a more complex scheme involving, for example, a second tumor suppressor locus in 9p21 distant from c5.1 and c5.3.

The observation that homozygous deletions of 9p21 occur in multiple tumor types suggests that the tumor suppressor gene(s) located there may be expressed in a wide variety of tissues. Thus, the proposed tumor suppressor gene(s) may be similar to the p53 gene in that it may participate in the development of multiple types of cancer. Other types of cancer have been reported in melanoma prone families, but whether the incidence of these cancers is statistically significant remains unclear (4, 17). A thorough deletion analysis of a wide variety of tumor types using c5.1 and c5.3 should help clarify the importance of this tumor suppressor gene in tumors other than melanoma.

Some of the homozygous deletions observed remove many genetic markers. Fountain *et al.* (18) reported that homozygous deletions of chromosome 9p21 in two different melanoma lines extended 2–3 megabases (18). In this study, at least one line, SK-MEL-5 contained deletions extending from the most distal marker tested, IFNA-1, past *D9S126*, a region apparently too large to be contained on a single YAC. The preponderance of large deletions suggests that the region surrounding *MLM* is devoid of genes that are essential to cellular viability.

CONCLUSION

A small region has been identified that is deleted in more than half of all melanomas. The simplest hypothesis is that this region contains a single gene, *MLM*, that controls genetic susceptibility to melanoma and possibly other cancers. In addition, *MLM* may be involved in the genesis and/or progression of tumors of sporadic rather than genetic origin. The region that contains *MLM* may be sparsely populated with genes. Thus, conventional screening procedures should suffice to identify candidates for the *MLM* gene within the region pinpointed by tumor deletion breakpoints.

Note Added in Proof. P1 names correspond to plate number, row, and well as follows: 109, 274E; 110, 273G; 140, 731H; 141, 1023F; 142, 1095C; 143, 877B; 144, 781E; 231, 994H; 232, 1447H; 233, 395A; 248, 677F; 249, 1288B; 349, 1069C; 364, 1184A; 428, 1073C; 452, 352E; 453, 725H; 508, 241H; 525, 555D; 526, 701A; 527, 829B; 731, 140H1; 732, 551D5; 816, 1216F7; 916, 608C9; 917, 1313C5; 921, 211H4; 1062, 49H4; 1063, 440E4; 1067, 271G11; 1068, 718B1; 1069, 801FA.

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