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Genetics

Genome-wide search for loss of heterozygosity in transgenic mouse tumors reveals candidate tumor suppressor genes on chromosomes 9 and 16

(allelotype/simple sequence length polymorphism/microsatellite/insulinoma/simian virus 40 large tumor antigen)

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ABSTRACT **A genome-wide scan for loss of heterozygosity (LOH) in tumors provides a powerful route to the identification of genes involved in tumorigenesis. This approach has not previously been applied to transgenic mice, despite the considerable advantages they afford for genetic dissection. Here, we report a genome-wide LOH analysis of insulinomas and carcinoid tumors in transgenic mice expressing the simian virus 40 large tumor oncogene. Although the overall genome-wide rate of LOH was quite low, chromosomes 9 and 16 showed high rates of allelic loss. About one-third of tumors showed partial LOH, allowing localization of the likely tumor suppressor genes to intervals of ≈11 centimorgans. The locus on chromosome 9, named *Loh-1*, lies in a region with synteny conservation to human chromosomes 3q, 6q12, 15q24, and 3p21, while the locus on chromosome 16, named *Loh-2*, lies in a region corresponding to human chromosomes 3q and 22q. Of particular note is the synteny conservation with human 3p21, which shows frequent loss in human cancers. These regions do not encode two tumor suppressors, pRB and p53, known to interact with large tumor oncoprotein, suggesting the presence of new genes whose loss of function contributes to multistage tumorigenesis.**

Loss of heterozygosity (LOH) studies have presented a powerful tool for the study of the development and progression of cancer (1). LOH analysis has been routinely employed to assess the status of particular genes in specific human tumor types and stages. It has also played an important role in positional cloning of oncogenes that were initially localized by other methods—including the genes for retinoblastoma (2, 3), Wilms tumor (4–6), and familial adenomatous polyposis (7–9). However, LOH has only been used in a few instances as a primary tool to search the entire human genome for loci involved in human tumorigenesis (10–16). Genome-wide scans for LOH in human tumors can be difficult to interpret for various reasons, including limitations on the number of tumors of a precise type and stage and potential differences in genetic background and environmental exposure among patients.

From a genetic standpoint, transgenic mice would appear to offer an ideal situation in which to perform genome-wide scans for LOH with the aim of genetic dissection of the process of tumorigenesis. A virtually unlimited supply of tumors of a specific stage can be obtained from genetically identical mice raised in a closely controlled environment. Moreover, one can construct transgenic mice carrying specific gene additions or knockouts to study the consequences of defined genotypes. With the recent availability of a genetic map of the mouse consisting of thousands of PCR-typeable simple sequence length polymorphisms (SSLPs) (17–20), LOH can be examined at many loci, even in small tumor samples, and the boundaries of each loss can be defined with high precision. In principle, by studying enough tumors, it should be possible to perform fine structure mapping and positional cloning of tumor suppressor genes.

Here, we report the results of a genome-wide LOH analysis of end-stage insulinomas and metastatic carcinoid tumors in transgenic mice expressing an oncogene, simian virus 40 large tumor antigen (Tag). Although the overall genome-wide rate of LOH was quite low, chromosomes 9 and 16 showed high rates of allelic loss, about one-third of which involved a partial LOH, consistent with the presence of single tumor suppressor loci, named *Loh-1* and *Loh-2*, on chromosomes 9 and 16, respectively.

MATERIALS AND METHODS

Tumor Purification and DNA Isolation. Primary pancreatic insulinomas and mesenteric lymph node metastases of small bowel carcinoid tumors were dissected free of adherent normal tissues. The tumor capsule was torn open and the tumor cells were gently dispersed with forceps; no enzymatic digestion was employed. The tumor cells were purified by gravity sedimentation in 10 ml of RPMI 1640 medium with 10% (vol/vol) calf serum, essentially as described (33). Vascular and stromal elements sediment in <1 min, while the tumor cells sediment in ≈10 min. Tumor cells were >90% pure as assessed by Wright–Giemsa staining and microscopic examination. DNA was isolated by deproteination overnight in 10 mM Tris·HCl, pH 8/10 mM EDTA/1% SDS/proteinase K (100 μg/ml) at 42°C, followed by phenol extraction and ethanol precipitation.

SSLP Genotyping. Genetic typing of SSLPs was performed by PCR with radioactively end-labeled PCR primer followed by electrophoresis on polyacrylamide gels. Primers were end-labeled with [γ-^{32}P]ATP (DuPont/NEN) according to standard protocols (22). A 20-ng aliquot of DNA was amplified as described (17). These PCR conditions were found to be within the linear range of amplification for loci tested. Electrophoresis and autoradiography were as described (17).

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Abbreviations: cM, centimorgan(s); SSLP, simple sequence length polymorphism; LOH, loss of heterozygosity; Tag, simian virus 40 large tumor antigen.

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LOH Analysis. SSLP loci were chosen to maximize the sensitivity and reliability of LOH analysis. The two SSLP alleles in (C57BL/6J × CAST)F_1 hybrids are not always of equal intensity, but the relative intensities are extremely reproducible among samples within an experiment and between experiments performed with the same template DNAs and under the same PCR conditions. Nonetheless, we tried to select SSLPs for which the intensities were relatively equal. LOH was detected by comparing tumor DNAs with at least two DNAs isolated from nontumor tissue of an F_1 animal. Subtle differences in the allelic ratio compared to these controls can easily be detected by eye. Quantitative analysis using a Fuji BAS2000 imaging system confirmed that LOH could be reliably seen in the presence of contamination with as much as 30% of normal F_1 DNA. Occasionally, a sample in one lane could interfere with the interpretation of a sample in an adjacent lane. In such cases, samples were reloaded with intervening lanes empty. Although the relative intensity of bands within a lane is consistent, the absolute intensity is not sufficiently consistent to allow one to distinguish between LOH due to hemizygosity resulting from deletion of an allele or LOH due to homozygous diploidy resulting from mitotic recombination or aberrant segregation.

The 76 SSLPs used in the initial screen were *D1Mit1, D1Mit11, D1Mit17, D2Mit1, D2Mit11, D2Mit25, D3Mit23, D3Mit46, D3Mit21, D3Mit4, D3Mit9, D3Mit13, D3Mit17, D3Mit19, D4Mit2, D4Mit9, D4Mit13, D5Mit1, D5Mit6, D5Mit51, D6Mit1, D6Mit9, D6Mit14, D7Mit21, D7Mit7, D7Nds4, D8Mit3, D8Mit5, D8Mit13, D9Mit1, D9Mit6, D9Nds2, D9Mit12, D9Mit19, D10Mit1, D10Mit8, D10Mit14, D11Mit16, D11Mit29, D11Mit31, D11Mit7, D11Mit11, D12Nds11, D12Mit2, D12Mit33, D12Mit3, D12Mit14, D12Mit6, D12Mit7, D12Mit16, D12Mit8, D13Mit44, D13Mit9, D13Mit30, D13Mit32, D14Mit1, D14Mit5, D14Mit9, D15Mit12, D15Mit5, D15Mit35, D16Mit9, D16Mit8, D16Nds2, D16Mit20, D17Mit7, D17Mit1, D18Mit19, D18Mit10, D18Mit5, D18Mit16, D19Mit3, D19Mit16, DXMit8, DXMit4*, and *DXMit12*. The additional SSLPs studied on chromosomes 9 and 16 are shown in Fig. 2.

RESULTS

Rip1–Tag2 Mice Develop Pancreatic Insulinomas and Intestinal Carcinoids. Transgenic mice carrying a hybrid oncogene composed of the insulin gene regulatory region aligned to control expression of the Tag oncoprotein reproducibly develop pancreatic islet tumors (insulinomas) of the insulin-producing β cells in multiple transgenic lineages (21). Statistical and histological analyses indicate the tumors arise through a multistep pathway in which expression of the oncogene is necessary, but not sufficient, to manifest a tumor: all of the β cells in the ≈400 pancreatic islets express Tag, initially without consequence; over time ≈50% of the islets show focal activation of β-cell hyperproliferation (23), which coincides with transcriptional activation of the insulin-like growth factor II gene (24); subsequently ≈10% of the islets progress to neovascularization (25); and finally 1–2% form solid tumors.

In one line of mice, Rip1–Tag2, metastatic intestinal carcinoid tumors develop concurrently with the pancreatic insulinomas (26). The carcinoid phenotype likely reflects a position effect of transgene integration, which in this line has been mapped to within 1 centimorgan (cM) of the preproglucagon gene locus on chromosome 2 (K. Smith and D.H., unpublished observations). The incidence of carcinoid tumors is low in Rip1–Tag2 mice inbred in C57BL/6J (≈5%) but can be dramatically enhanced (up to ≈100%) when these mice are crossed to another transgenic line (26) or to other inbred mouse strains, including A/J and SCID (G. Christofori, F. Radvanyi, and D.H., unpublished observations), and *Mus musculus castaneus* (this study).

To assess LOH, it is best to study mice that are heterozygous at many loci across the genome. Accordingly, we produced F_1 hybrid mice between the Rip1–Tag2 transgenic line (with the transgene having been made congenic on the C57BL/6J background) and the widely divergent subspecies *M. m. castaneus* (CAST/Ei). These F_1 animals developed islet β-cell tumors with the same frequency, latency, and pattern of progression as the original Rip1–Tag2 transgenic line. In addition, intestinal carcinoids arose in ≈60% of these F_1 mice. Solid islet tumors and intestinal carcinoid tumors metastatic to mesenteric lymph nodes from these F_1 animals were harvested, tumor cells were purified from surrounding stroma, and DNA was extracted. Because the tumors yielded at most 5 μg of DNA, it was necessary to use PCR-typeable genetic markers to study LOH. There is now a dense genetic map of the entire mouse genome, containing >4000 PCR-typeable SSLPs, each of which can be genotyped using only 20 ng of genomic DNA (20). Based on reconstruction experiments, we determined that these SSLPs could be reliably used to detect allelic losses in DNA samples that were contaminated with as much as 30% of normal F_1 DNA. In tumors, LOH was evident from either complete absence or greatly decreased intensity of an allele compared to control F_1 DNA (Fig. 1).

Initial Genome-Wide Screen. As an initial genome-wide screen for LOH, DNAs from 22 islet cell tumors were genotyped with 76 SSLP markers distributed over the entire genome, with at least 2 markers per chromosome. Although LOH can occur at random in tumor cells, the overall frequency of chromosomes showing LOH was low (4.5%)—with a typical chromosome showing allelic loss in at most one tumor (Table 1).

LOH was found in this initial screen for a contiguous set of markers in 13 of 20 islet tumors and for a single marker in the remaining 7 islet tumors. In the latter case, we carefully reconfirmed the results and also studied nearby markers to see if a larger region of LOH could be identified. To test whether the use of a denser genetic map would reveal more LOH events, we studied a collection of 9 loci distributed at a spacing of 6 cM along chromosome 12. The denser sampling did not identify any additional LOH events, suggesting that our initial sampling with two or three markers per chromosome was sufficient to detect most events.

LOH on Chromosomes 9 and 16. Although the genome-wide rate of LOH was low, chromosomes 9 and 16 showed significantly high rates of 18 and 32%, respectively, in the initial survey of 22 insulinomas (Table 1). To pursue these results, we genotyped DNAs from an additional 43 insulino-

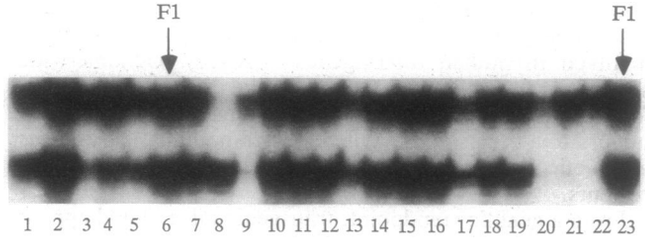

FIG. 1. LOH analysis of insulinoma DNA samples. Example of genotyping of the locus *D16Mit20* on DNAs from 21 insulinomas, with two normal F_1 DNA samples (indicated) as controls. Radioactive PCR products were electrophoresed on a denaturing 7% polyacrylamide gel and exposed to x-ray film. Lanes 4, 5, 8, 9, 20, 21, and 22 show clear LOH. Lane 3 appears to show LOH but, because it was adjacent to two lanes with stronger signals, was reconfirmed by repeating the electrophoresis with adjacent lanes empty. The larger allele (upper band) is C57BL/6J; the smaller allele (lower band) is CAST.

Table 1. Initial screen for LOH in 22 insulinomas

Chromosome	Markers, no.	% tumors with LOH
1	3	4.5 (1/22)
2	3	0.0 (0/22)
3	8	4.5 (1/22)
4	3	4.5 (1/22)
5	3	0.0 (0/22)
6	3	0.0 (0/22)
7	3	0.0 (0/22)
8	3	9.1 (2/22)
9	5	18.2 (4/22)*
10	3	0.0 (0/22)
11	5	4.5 (1/22)
12	9	4.5 (1/22)
13	4	4.5 (1/22)
14	3	0.0 (0/22)
15	3	0.0 (0/22)
16	4	32.0 (7/22)†
17	2	4.5 (1/22)
18	4	0.0 (0/22)
19	2	0.0 (0/22)
X	3	0.0 (0/22)

Percentage of tumors with LOH at any marker along chromosome is shown. Numbers in parentheses are number found/number examined.

*Difference from background rate (4.5%), significant at nominal P = 0.013 level.

†Difference from background rate (4.5%), significant at nominal P = 0.00003 level.

mas for genetic markers on these two chromosomes (Table 2). The rate of LOH on chromosomes 9 and 16 remained similar in the overall sample, being 20 and 32%, respectively. Both rates were highly significantly different than the background genome-wide rate ($P < 0.0001$). In addition, LOH analysis of 20 carcinoid tumors showed a similar pattern of a low overall rate across the genome, with significantly high rates (15%) on chromosomes 9 and 16.

These data strongly suggest that both chromosomes 9 and 16 contain a gene or genes in which somatic mutations are important for the progression of Tag oncogene-induced neoplasms to solid tumors (1, 27, 28). To localize these genes more precisely, we examined those tumors that showed partial LOH on these chromosomes. Although the majority of LOH events on chromosomes 9 and 16 showed allelic loss along the entire chromosome, partial losses were seen in 40% of the LOHs on chromosome 9 and in ≈25% on chromosome 16 (Table 2). By genotyping a dense collection of genetic markers, the breakpoints for LOH could be defined to within a few centimorgans. A consistent pattern of LOH was found for both chromosomes, defining a smallest region of overlap of 11–12 cM (Fig. 2). Based on the consistency of the LOH pattern, we presume that each region contains a gene involved in tumor initiation or progression. We refer to the genes on chromosomes 9 and 16, respectively, as *Loh-1* and *Loh-2*.

Pattern of Allelic Loss. One advantage of using genetically defined mice for LOH studies is that it is easy to test for biases in the spectrum of somatic mutation that would suggest either the presence of imprinting or the presence of a susceptibility allele in one parental strain. There was no significant bias in the parental origin of the chromosome showing LOH on either chromosome 9 or 16, indicating no effect of genetic imprinting (Table 3). There was also no bias in the particular allele lost at *Loh-1* (Table 3). In contrast, there was a highly significant bias in the particular allele lost at *Loh-2*, with the CAST allele lost in >80% of cases ($P < 0.002$). This suggests a functional difference between *Loh-2* alleles that favors those tumor cells that lose the function of the CAST allele.

Finally, we found no correlation between the occurrence of allelic losses at *Loh-1* and *Loh-2*. The observed frequency of tumors with losses on both chromosomes 9 and 16 is not significantly different than would be expected by chance if the losses occurred independently (5% vs. 6%). Both insulinomas and carcinoid tumors showed these two losses, suggesting a commonality among these neuroendocrine cell types or in the tumorigenesis pathway elicited by the Tag oncoprotein.

DISCUSSION

LOH analysis provides a way to recognize a subset of the somatic and germ-line mutations involved in the initiation and onset of cancer. This technique has been extensively used in humans to study mutational events at known loci and has played a key role in positional cloning of candidate genes identified by other methods. Recently, genome-wide LOH analysis has been undertaken in several human tumor types to identify loci involved in human tumor initiations and progression (10–16).

In contrast to LOH studies in humans, the use of laboratory mice allows the study of an unlimited number of tumors of a defined stage and type, eliminates the problem of genetic heterogeneity, and facilitates the recognition of imprinting effects and heritable susceptibility factors (through nonrandom distributions of the allele lost) (29, 30). Although LOH studies in transgenic mice are not immediately relevant to

Table 2. Allelic losses on chromosomes 9 and 16

		Tumor																													
		10T3	42T3	36T2	1T2	60T4	18T1	57T2	31A	70T2	T2B	10T1	00T2	55T1	55T4	66T1	66T3	44T2	77T4	88T2	88T3	66T1	66T3	66T4	66T5	66T6	44T3	88T5	00T3	00T4	11T3	11T2
Chr.	SSLP																														
9	Mit1			C		B	B			C		C			C						C		B				B				
	Mit6			C		B	B			C		C			C			B			C		B				B				
	Nds2			C		B	B			C		C	B		C			B			C		B				B				
	Mit12			C		B	B			C		C	B	B	C	B		B			C		B				B				
	Mit19			C		B	B			C		C	B	B		B		B			C		B				B				
16	Mit8	C	C	B	C			C	C	C							B		B	C	C	C		C	C		C			C	
	Nds2	C	C	B	C			C	C	C	C						B		B	C	C	C		C	C	C	C		B	C	C	C
	Mit20	C	C	B	C			C	C	C	C						B		B	C	C	C		C	C	C	C		B	C	C	C

Sixty-five tumors were analyzed; only the 31 tumors (10T3 to 71T2) that displayed LOH on chromosomes 9 and/or 16 are shown here. The first 9 samples are from the 22 tumors analyzed for LOH across the entire genome. The last 22 are from the group of 43 tumors studied only with markers on chromosome 9 and 16. B, LOH of the C57BL/6 allele; C, LOH of the CAST allele. The LOH rate in this second set is highly significant on chromosome 9 (9 of 43 tumors; $P = 0.0001$) and chromosome 16 (14 of 43 tumors; $P = 3 \times 10^{-9}$). Chr., chromosome.

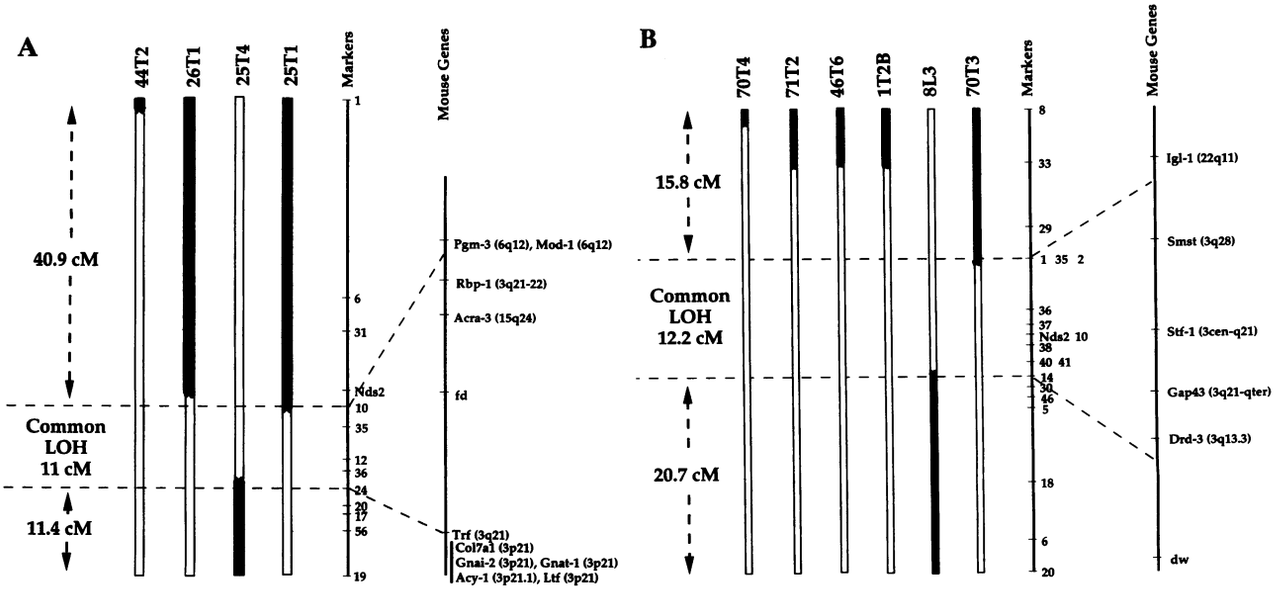

FIG. 2. Mapping of *Loh-1* and *Loh-2*. Individual tumors showing partial LOH, indicated by open boxes, are shown on left. Genetic maps showing markers used for LOH analysis are shown in the center. The names of the markers omit the prefix *DNMit*, where *N* indicates the chromosome number. Genetic maps showing the location of known mouse genes are shown to the right, with approximate cytogenetic location of human homologues, where known, indicated in parentheses. The smallest region of overlap is indicated by dashed horizontal line. Solid boxes indicate retention of heterozygosity. (*A*) Chromosome 9. (*B*) Chromosome 16.

human clinical medicine, they may prove to be the most effective way to dissect the biological pathway of tumorigenesis. With the availability of scores of transgenic mouse lines carrying gene insertions and knockouts, it is possible to manipulate the genome to define the somatic mutations seen in mice carrying particular mutations or combinations of mutations conferring susceptibility to specific tumor types.

In the present study, we found strong evidence for two genes, *Loh-1* and *Loh-2*, on chromosomes 9 and 16, respectively, involved in insulinomas and carcinoid tumors that develop in transgenic mice carrying an insulin promoter joined to the coding information for the Tag oncoprotein. The Tag oncoprotein is remarkably effective at eliciting tumorigenesis in transgenic mice, with at least 30 diverse cell types demonstrably transformed by its expression (31). Conventional wisdom argues that the potency of Tag lies in its ability to bind and inactivate two known tumor suppressors, p53 and pRB (32). These two genes are located on mouse chromosomes 11 and 14, respectively, neither of which suffered LOH in the present study. Indeed, it appears that p53 is bound to Tag and sequestered throughout the tumorigenesis pathway in Rip1–Tag2 mice (33). We infer there is no need to genetically inactivate p53; nevertheless, its loss of function as a "guardian of the genome" may allow critical mutations, including the observed LOH, to arise. Thus, our data suggest that there are other genes whose loss or attenuation of function contributes to some important aspect of the tumor phenotype, which includes not only tumor cell proliferation but also induction of angiogenesis. Given the wide range of tumors that have been induced by various transgenes expressing Tag [including insulinomas, hepatomas, pituitary somatotroph tumors, rhabdomyosarcomas, osteosarcomas, pheochromocytomas, retinoblastomas, atrial cell tumors, and lens cell tumors (31)], it will be particularly interesting to determine whether the same LOH pattern is seen in all of the various transgenic lines expressing Tag or whether the pattern varies with the tumor type.

Table 3. Origin of alleles lost on chromosomes 9 and 16 in tumors

	Parental alleles lost, no.		Strain of allele lost, no.	
Chr.	Maternal	Paternal	C57BL/6J	CAST
9	8	8	8	8
16	14	10	4	20

Data are based on 65 insulinomas and 20 carcinoid tumors. Chr., chromosome.

Mouse LOH studies should have the greatest impact if they permit localization of tumor suppressor genes to small enough intervals to allow positional cloning—thereby, allowing molecular studies on their contributions to tumorigenesis and their possible relevance to human cancers. In fact, it was possible to map these genes to regions of ≈11 cM based on the LOH pattern among 85 tumors. By studying a larger collection of tumors, it should be possible to map these genes more precisely. It should only be necessary to genotype each tumor for two loci flanking *Loh-1* and *Loh-2* to identify the subset having partial LOH with a breakpoint in the critical region of LOH. These tumors could then be genotyped for a denser collection of genetic markers across the region. If breakpoints are random, a survey of 1000 tumors should allow rapid localization to ≈1 cM, which could be sufficient for positional cloning. Treatment of mice with x-rays or other mutagens might increase the frequency of partial LOH and further improve the resolution of the mapping.

Even in advance of positional cloning of *Loh-1* and *Loh-2*, some information can be gleaned about the likely position of the human homologues based on synteny conservation (34) between mouse and human genomes (Fig. 2). The human homologues of genes near *Loh-1* have been mapped to chromosomes 3q, 6q12, 15q24, and 3p21 (several genes mapping to the latter region have not been precisely mapped relative to the breakpoints defining the region of LOH), while the homologues of genes near *Loh-2* have been mapped to human chromosomes 3q and 22q11 (Fig. 2). Of these regions, chromosome 3p21 is particularly notable because it is a hot spot for LOH in a wide variety of human tumor types (35). The other regions of homology have not been implicated in human tumors.

An interesting observation about *Loh-2* is the preferential loss of the CAST allele in tumors. This suggests that there may be a heritable difference between the B6 and CAST

alleles, with the CAST conferring some measure of protection against tumorigenesis. If so, *Loh-2* might be expected to act as a heritable modifier of tumor risk in a (B6 × CAST)F_2 intercross (36). It will be interesting to examine the LOH pattern in F_1 hybrids with other mouse strains. The CAST strain was originally chosen because the rate of SSLP with laboratory strains is especially high. However, it is feasible to use virtually any combination of strains because the rate of polymorphism among laboratory strains is still ≈50% (17).

This study has demonstrated the feasibility of combining the reproducibility and genetic manipulability of a transgenic mouse model with a powerful technology for monitoring changes in the genome to identify candidate genetic loci involved in tumorigenesis. The last 10 years have seen numerous reports of transgenic mouse models of cancer involving both dominant oncogenes and inactivated tumor suppressor genes (37), in general involving multistage pathways that implicate additional genetic or epigenetic changes. There has been persistent optimism that these models will prove of value in elucidating general principles of tumor development relevant to mechanisms of human cancer. It can be anticipated that application of this genome scanning technique will make a significant contribution to the utility of transgenic mouse models as systems to identify, describe, and eventually interfere with genetic mechanisms of multistage tumor development.

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