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Mouse mammary tumor virus infection accelerates mammary carcinogenesis in *Wnt-1* transgenic mice by insertional activation of *int-2/Fgf-3* and *hst/Fgf-4*

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ABSTRACT **Transgenic mice carrying the *Wnt-1* protooncogene modified for expression in mammary epithelial cells exhibit hyperplastic mammary glands and stochastically develop mammary carcinomas, suggesting that additional events are necessary for tumorigenesis. To induce such events and to identify the genes involved, we have infected *Wnt-1* transgenic mice with mouse mammary tumor virus (MMTV), intending to insertionally activate, and thereby molecularly tag, cooperating protooncogenes. Infection of breeding female *Wnt-1* transgenics decreased the average age at which tumors appeared from ≈4 months to ≈2.5 months and increased the average number of primary tumors per mouse from 1–2 to >5. A smaller effect was observed in virgin females, and infection of transgenic males showed no significant effect on tumor latency. More than half of the tumors from the infected breeding group contained one or more newly acquired MMTV proviruses in a pattern suggesting that most cells in tumors arose from a single infected cell. Analyses of provirus-containing tumors for induced or altered expression of *int-2/Fgf-3*, *hst/Fgf-4*, *int-3*, and *Wnt-3* showed activation of *int-2* in 39% of tumors, *hst* in 3%, and both *int-2* and *hst* in 3%. DNA analyses with probes for protooncogenes and MMTV confirmed that the activations resulted from proviral insertions. There was no evidence for proviral insertions at the *int-3*, *Wnt-3*, or *Wnt-1* loci. These findings provide further evidence that fibroblast growth factors Int-2 and Hst can cooperate with Wnt-1, another secreted factor, in mammary tumorigenesis, and they illustrate the capacity of this system to identify cooperating oncogenes.**

The oncogenic potential of *Wnt-1* (formerly *int-1*) was initially inferred from its frequent transcriptional activation by the nearby insertion of mouse mammary tumor virus (MMTV) proviruses in mammary tumors of infected mice (1). Expression of *Wnt-1* in mouse mammary epithelial cell lines imparts some characteristics of transformation (2, 3); however, the generation of mice carrying a *Wnt-1* transgene under the influence of a MMTV enhancer was required to provide firm proof of the oncogenic effects of *Wnt-1*. Both male and female transgenic mice develop mammary adenocarcinomas and, less frequently, salivary gland adenocarcinomas (4).

The median latency of mammary tumor formation in female *Wnt-1* transgenics is ≈5 months of age, with >80% of mice developing tumors by the age of 7 months (4). Males develop tumors later in life and less frequently. *Wnt-1* transgenic mice of both sexes also display a marked hyperplasia of the mammary gland with extensive lobular alveolar development. This mammary hyperplasia, coupled with the delayed kinetics and sporadic nature of tumor development, argues that *Wnt-1* contributes to, but is not sufficient for, tumorigenesis in these mice. Other events, presumably genetic, are necessary for progression to neoplasia.

In an attempt to identify genes that may be involved in multistep tumorigenesis in this model system, we have mutagenized *Wnt-1* transgenic mice by infection with MMTV, predicting that the resulting tumors will contain insertionally activated protooncogenes that cooperate with *Wnt-1*. This approach has an advantage over other mutagenesis procedures in that tumors arising due to proviral insertions contain proviruses physically linked to activated protooncogenes. This permits identification or cloning of the activated genes, as has been demonstrated in other transgenic animals infected with murine leukemia viruses (5–7).

We report that MMTV accelerates mammary tumorigenesis in *Wnt-1* transgenic animals—especially in breeding females. By examining the tumors for the involvement of five protooncogenes that are known targets for MMTV insertion mutations, we have found that two fibroblast growth factor (FGF) genes, *int-2/Fgf-3* and *hst/Fgf-4*, are activated in tumors by proviral insertion.

MATERIALS AND METHODS

Mice and Viral Infections. The transgenic and nontransgenic mice used in this study were the offspring generated from crosses of *Wnt-1* transgenic males from line 303 (4) with BALB/c females or from subsequent backcrosses of F_1 transgenic male offspring with BALB/c females. Mice were infected with MMTV at 3–4 weeks of age by intraperitoneal injection of 10^7 EH-2 cells and were examined at weekly intervals for the appearance of tumors. The EH-2 cell line is a clone of rat XC cells producing a pathogenic hybrid MMTV that consists primarily of MMTV(C3H) sequences (8).

Nucleic Acid Isolation, Analysis, and Hybridization Probes. DNA was isolated from tumors and normal tissues and hybridized as described (8). Total cellular RNAs were extracted from cells by the urea/LiCl method (9), electrophoresed through 1% agarose formaldehyde gels, and hybridized with DNA probes (8). Probes were removed by treatment with 0.1% SDS at 95°C.

The MMTV probes were a 1.9-kb *Pst* I/*Xho* I fragment (*gag* probe) and a 1.2-kb *Bam*HI fragment (*env* probe) from the hybrid MMTV clone (8). Two *int-2* probes were used: a 1.3-kb fragment from the 5′ end of cDNA clone c.28 to the *Eco*RI site in a region of exon 3 (10), and a 1.7-kb fragment derived from the exon 3 portion of the cDNA clone c.3L by cleavage with *Bam*HI (10). The *hst* probes were genomic *Hin*dIII fragments of 1.6 (HH1) and 4 (HH3) kb as shown in Fig. 6 and ref. 11. Probe J is a 2.1-kb *Sac* I fragment of genomic mouse DNA that can detect rearrangements in both

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Abbreviations: FGF, fibroblast growth factor; MMTV, mouse mammary tumor virus.

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int-2 and *hst* (11). The *Wnt-3* probe was a 1.7-kb *Eco*RI fragment derived from clone pCG1 (12). The *int-3* probes were a genomic *Pst* I 0.7-kb fragment from pC241L and three genomic fragments from this locus called probes A, B, and G (13). The *Wnt-1* probe was a 2.1-kb cDNA fragment derived from clone 26 (14).

RESULTS

MMTV Infection Accelerates Tumorigenesis in *Wnt-1* Transgenic Mice. Our strategy to identify protooncogenes that collaborate with *Wnt-1* during mammary tumorigenesis was to activate (and molecularly tag) these genes by MMTV proviral insertion after infection of *Wnt-1* transgenic mice. Activation of a gene that cooperates with *Wnt-1* and thus confers a growth advantage would presumably produce a tumor composed mainly of cells clonally derived from the cell that acquired the proviral insertion.

If infection accelerates secondary events required for the appearance of mammary tumors, then tumors would be expected to arise earlier in infected than in uninfected animals. To test for this effect, we infected three groups of *Wnt-1* transgenics (males, virgin females, and breeding females) at 3–4 weeks of age with a pathogenic strain of MMTV (8), and we monitored these animals and parallel groups of uninfected transgenics for mammary tumors. In addition, MMTV-infected and uninfected nontransgenic littermates—again males, virgin females, and breeding females—were included as controls. Breeding and virgin females were analyzed separately, since breeding may stimulate viral spread and tumor induction by hormonal effects on epithelial cell growth (15).

We observed no significant change in the frequency or time of onset of tumors in infected male transgenics compared to uninfected animals; as expected, no tumors were found in infected or uninfected nontransgenic males (Fig. 1*A*). In contrast, tumor formation was augmented by MMTV infection of virgin or breeding female transgenics (Fig. 1 *B* and *C*). In the breeding group, which showed the more pronounced effects, the median time from birth to detection of tumors decreased from ≈4 months to ≈2.5 months as a result of infection. Infection of nontransgenic females induced mammary tumors with extended latencies (8–10 months) and a strong dependence on breeding (Fig. 1 *B* and *C*). Uninfected, nontransgenic female littermates, whether virgin or breeding, did not develop tumors over the course of 12 months, confirming that the mice did not harbor pathogenic MMTV. In the aggregate, the data presented in Fig. 1 are consistent with the notion that MMTV can accelerate mammary tumorigenesis in mice with a preexisting *Wnt-1* transgene, presumably by proviral insertions that activate protooncogenes.

In addition to the effect of MMTV infection on tumor latency, we observed a dramatic increase in the number of mammary tumors in virus-infected *Wnt-1* transgenic females (Fig. 2). At 4 months of age, infected transgenic breeding females showed an average of >5 mammary tumors per mouse, with some animals developing 10 tumors—1 in each mammary gland. Uninfected transgenic animals, however, rarely developed >1 tumor and never developed >3. Multiple tumors from infected mice appear to arise independently and not as a result of metastases, as shown by analysis of proviral junction fragments (see below; Fig. 3). The increase in number of tumors further suggests that MMTV infection augments tumorigenesis in *Wnt-1* transgenic females.

Tumors from Infected *Wnt-1* Transgenics Contain MMTV Proviruses. To identify those tumors likely to contain activated protooncogenes, we screened mammary tumor DNAs from MMTV-infected *Wnt-1* transgenic females for proviral insertions. Tumors from breeding female transgenics were chosen because of the stronger effect of infection on tumor

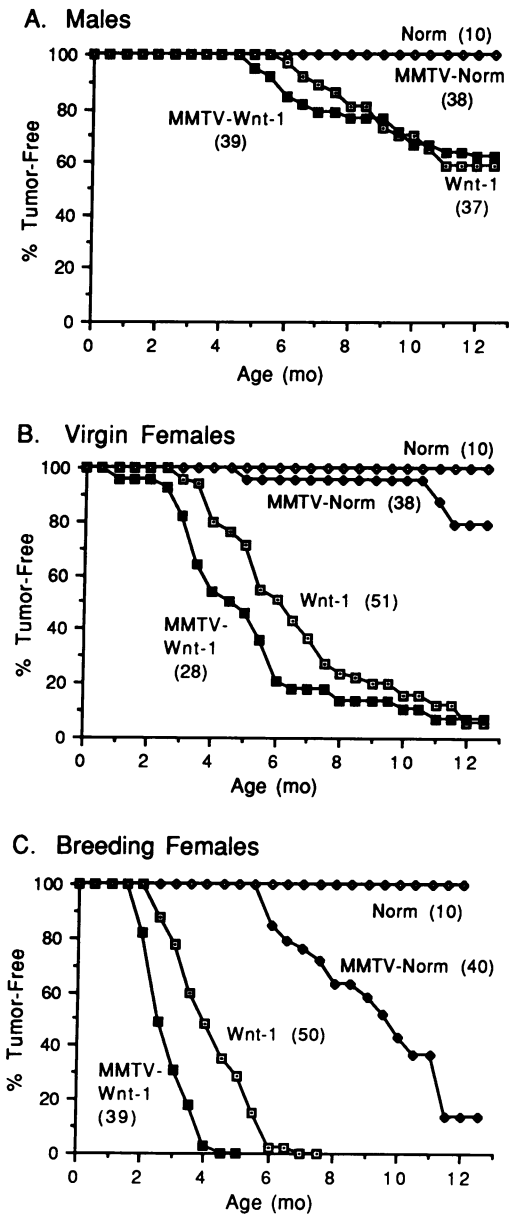

FIG. 1. Acceleration of mammary tumorigenesis in MMTV-infected *Wnt-1* transgenic mice. Percentage of mice in each group remaining free of palpable mammary tumors is plotted as a function of age. MMTV-infected animals received virus at 3–4 weeks of age. (*A*) Males. (*B*) Virgin females. (*C*) Breeding females. Wnt-1, *Wnt-1* transgenics; MMTV-Wnt-1, MMTV-infected *Wnt-1* transgenics; Norm, nontransgenics; MMTV-Norm, MMTV-infected nontransgenics. Numbers of animals in each group are shown in parentheses.

latency in these mice. The analysis was performed with restriction enzymes that cut proviral DNA, so that our probes for viral DNA should detect host–viral junction fragments. Since MMTV DNA can integrate into many sites in the mouse genome, only those proviruses present in a large proportion of the cells in a tumor will be scored by this method. A positive result implies that the tumor is composed, at least in part, of a clone of MMTV-infected cells.

Southern blot analysis of *Eco*RI-digested tumor DNAs, tested with a hybridization probe from the *gag* region of MMTV to detect junction fragments from the 5′ ends of proviruses, showed that at least 75 of 128 tumor DNAs (59%) contained proviruses in a clonal or semiclonal pattern (for examples, see Fig. 3). In addition, multiple tumors from a single mouse appeared to be of independent origin since junction fragments are unique to each tumor (Fig. 3). The

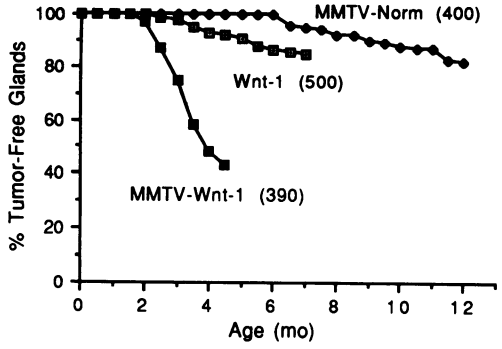

FIG. 2. Increased numbers of mammary tumors per mouse after MMTV infection. All 10 glands in three cohorts of breeding female mice—MMTV-infected nontransgenics, uninfected *Wnt-1* transgenics, and infected *Wnt-1* transgenics—were monitored for the appearance of tumors. Graph indicates percentage of glands remaining tumor-free at the indicated ages. Abbreviations are the same as in Fig. 1. Numbers of glands in each group are shown in parentheses.

actual number of tumors with such proviruses is undoubtedly higher, since some *Eco*RI-generated junction fragments comigrate with endogenous MMTV proviral fragments. A small sampling of tumor DNAs that appeared to be provirus negative by *Eco*RI analysis revealed acquired proviruses in some cases when analyzed with *Xho* I (data not shown).

Transcriptional Activation of *int-2* and *hst*. Tumors identified as containing proviral insertions in a clonal pattern were considered candidates for harboring MMTV-activated protooncogenes. Activation of protooncogenes by MMTV proviral insertion has been shown to occur in one of two ways: by augmented transcription (11, 12, 16, 17) or by truncation of a coding domain (18). Sixty-four of the tumors with clonal insertions were tested for such mutations of selected protooncogenes by Northern blot analysis of total cellular RNAs. The protooncogenes *int-2*, *hst*, *int-3*, and *Wnt-3* were chosen since these genes, in addition to *Wnt-1*, have previously been shown to be targets for MMTV insertional mutagenesis (1, 11–13, 19). The high level of *Wnt-1* RNA in transgenic mammary glands (4) precluded tests for insertion activation of endogenous *Wnt-1* genes by Northern blotting.

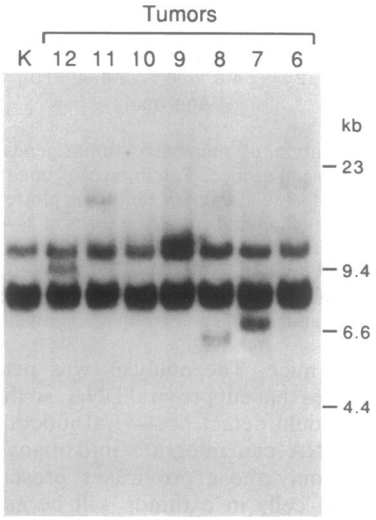

FIG. 3. MMTV proviruses in tumors of infected *Wnt-1* transgenic mice. DNAs isolated from a kidney (K) and seven mammary tumors from a single MMTV-infected, breeding *Wnt-1*-transgenic female mouse were digested with *Eco*RI, separated by gel electrophoresis, blotted to nylon membranes, and hybridized with a ^{32}P-labeled MMTV *gag* probe. In addition to the endogenous MMTV proviruses, proviruses specific for most tumors are detected as fragments of unique size, indicating independent clonal or semiclonal populations of infected cells. *Eco*RI cleaves once within the genome of the MMTV used for infection (8).

No abnormal transcripts from the *int-3* or *Wnt-3* loci were detected in tests of RNA from 64 tumors (data not shown). However, both *int-2* and *hst*, two genes in the FGF family not normally expressed in mouse mammary glands, were found to be transcriptionally active in many of the tumors. Of the 64 tumors tested with proviral insertions, *int-2* RNA was present in 25 (39%), *hst* RNA was present in 2 (3%), and both *int-2* and *hst* RNA were present in 2 (3%) (Fig. 4). *int-2* and *hst* RNAs were not detected in the remaining 35 tumors (55%) (data not shown). The abnormally large *int-2* transcript(s) in tumor 126 (Fig. 4) probably initiate from the inserted provirus located upstream and in the same orientation as *int-2* (see below and Fig. 6).

MMTV Proviral Insertion into *int-2* and *hst* Loci. To confirm that *int-2* and *hst* RNAs observed by Northern blotting were indeed produced as a consequence of MMTV proviral insertions, we tested tumor DNAs for proviruses in these two closely linked genetic loci. Viral–host junction fragments that appeared to hybridize to probes both for the locus of interest and for MMTV were detected in the majority of tumors that expressed these genes (Fig. 5; data not shown). From the DNA blotting results, it was possible to deduce the position and orientation of each provirus (Fig. 6). Importantly, none

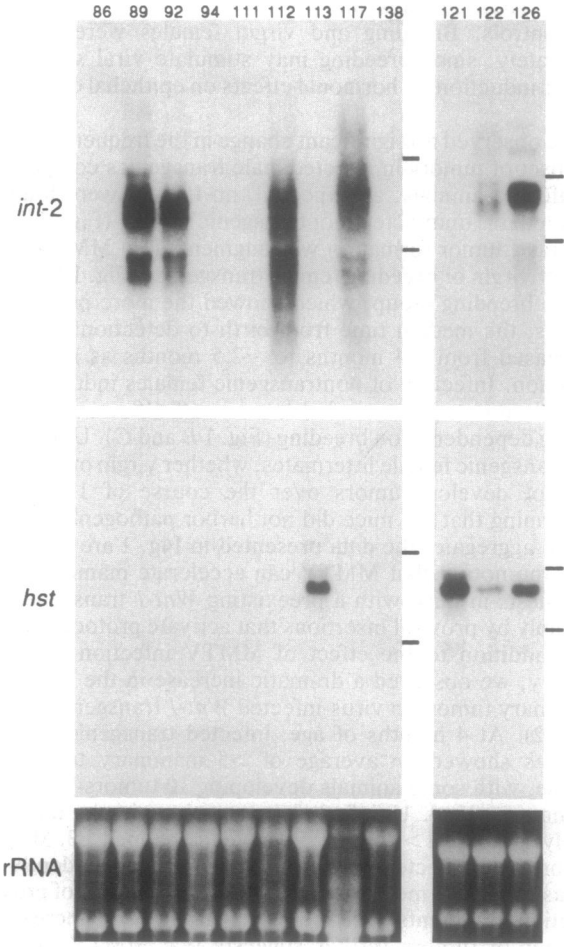

FIG. 4. Transcriptional activation of *int-2* and *hst* by MMTV insertion in tumors from infected *Wnt-1* transgenic mice. Representative Northern blots of total cellular RNAs from tumors were hybridized with ^{32}P-labeled probes for *int-2* or *hst*. Bars on the right indicate positions of 28S and 18S rRNAs, as displayed in the ethidium bromide-stained gel (*Lower*). Note activation of both *int-2* and *hst* in tumors 122 and 126.

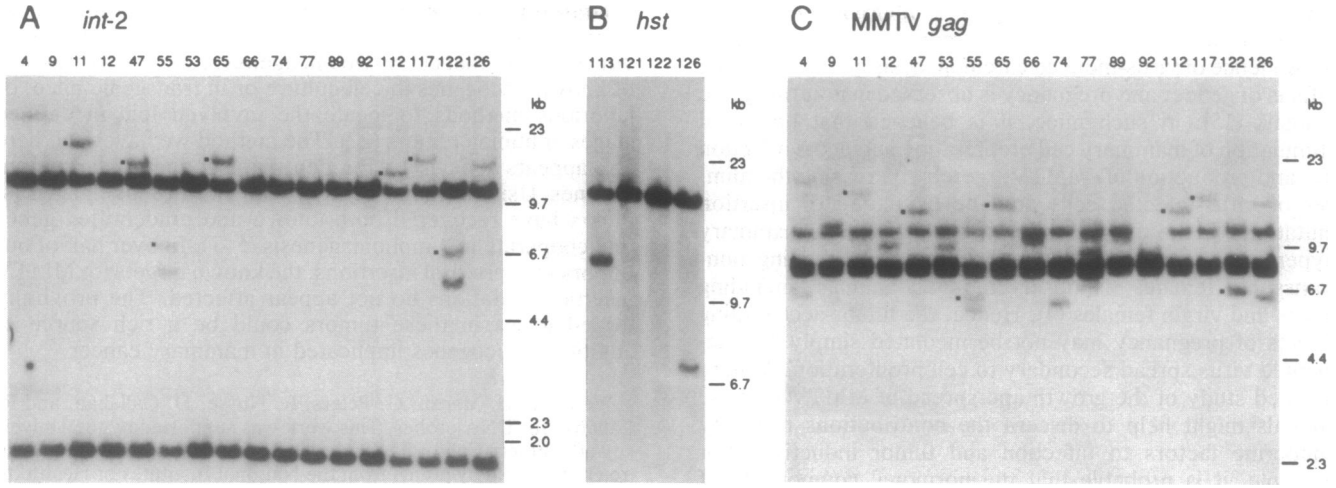

FIG. 5. Mapping of MMTV proviral insertions in *int-2* and *hst* loci. Rearrangements of the *int-2* and *hst* loci due to MMTV insertions were detected in Southern blot analyses of restriction enzyme-digested tumor DNAs using ^{32}P-labeled probes for *int-2* (*A*), *hst* (*B*), or *gag* region of MMTV (*C*). DNAs in *A* and *C* were digested with *Eco*RI, and those in *B* were digested with *Bam*HI. Bands detected at similar positions after annealing with both *int-2* and MMTV probes are marked by dots in *A* and *C*. Probe for *int-2* was a mixture of clones c.28 and c.3L, and probe for *hst* was probe J (see Fig. 6).

of the mapped insertions appeared to interrupt the coding domains of *int-2* or *hst*, consistent with previous results in MMTV-induced tumors in nontransgenic mice (11, 17), suggesting that the production of intact proteins from the activated loci is essential for tumorigenesis. In addition, proviruses were nearly always upstream of the coding sequence of the affected gene in the opposite transcriptional orientation or downstream of the coding sequence in the same orientation. We could not detect proviral insertions in *int-2* in some tumors containing *int-2* RNA, a difficulty also reported by others using nontransgenic mice (16, 20–22). This could be due to the limited size of the tested chromosomal domain, the comigration of junction fragments with fragments from normal alleles, insertions affecting a minority of cells, or other modes of activation of *int-2*. Tumor DNAs were also examined for insertions into *int-3*, *Wnt-3*, and *Wnt-1*, but no rearrangements of these loci were observed (data not shown).

Two tumors, 122 and 126, contained both *int-2* and *hst* RNAs. In tumor 122, one MMTV provirus located between the two genes presumably accounts for activation of both loci. Surprisingly, two proviruses were found in tumor 126; one was located 5′ of *int-2* in the same transcriptional orientation as the gene, and the other was located 5′ of *hst* in the opposite orientation (Fig. 6). This finding raises the possibility that *int-2* and *hst* can cooperate with each other, as well as with *Wnt-1*, during tumorigenesis, but other explanations are also possible (see *Discussion*).

DISCUSSION

We have used insertional mutagenesis of *Wnt-1* transgenic mice by MMTV to activate and identify protooncogenes that cooperate with *Wnt-1* in mammary tumorigenesis. Infection resulted in accelerated tumor formation and an increased number of tumors per mouse in female transgenics, especially in breeding animals. Analysis of mammary tumors showed that most tumor DNAs possessed proviral insertions in a clonal pattern. In almost half of the tumors with insertions, the *int-2* or *hst* protooncogene was transcriptionally activated, suggesting collaboration with *Wnt-1*.

Infection of *Wnt-1* transgenic animals with MMTV had the most dramatic effects in breeding females, but produced only

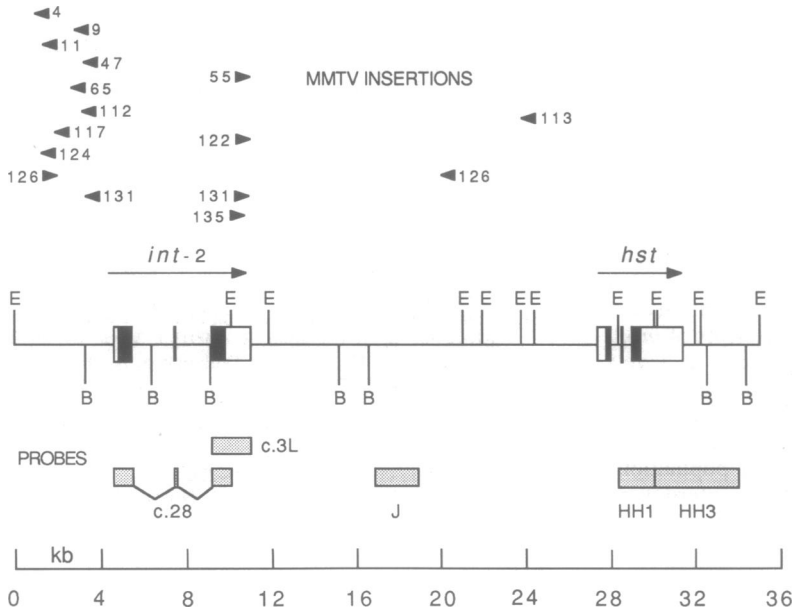

FIG. 6. Map of MMTV proviral insertions into the *int-2*/*hst* region. Proviral insertions were mapped from Southern blot data using probes from this region and from MMTV. Arrowheads denote location and transcriptional orientation of MMTV proviruses. (Note that 5′ ends of proviruses are preferentially oriented closest to the activated gene and coding domains of *int-2* and *hst* are not interrupted by insertions.) E, *Eco*RI; B, *Bam*HI.

a modest acceleration of tumorigenesis in virgin females, and did not affect the appearance of tumors in males. A similar dependence of virus-induced carcinogenesis on the hormonal effects of gender and pregnancy is observed in nontransgenic animals (15); in such mice, it is believed that hormonal stimulation of mammary cell proliferation augments infection by, and production of, MMTV, thereby increasing the number of virus-infected cells and the likelihood of insertion mutations. In the *Wnt-1* transgenic mice, however, mammary hyperplasia, similar to that encountered in breeding nontransgenic females, is observed in all animals, including males and virgin females (4). Hence, the tumor-accelerating effects of pregnancy may not be mediated simply by augmented virus spread secondary to cell proliferation. A more detailed study of the growth and spread of MMTV in these animals might help to discern the contributions made by endocrine factors to infection and tumor induction. For example, it is probable that the hormonal components of pregnancy and lactation have a direct effect on tumorigenesis and do not simply promote spread of MMTV, as is apparent from the hormone-dependent growth of some murine mammary tumors (22, 23).

The presumed oncogenic collaboration of *Wnt-1* with *int-2* or *hst* in the mice described here is supported by several kinds of evidence: acceleration of tumorigenesis in animals infected by MMTV; insertions into the *int-2* and *hst* loci, with coordinate appearance of *int-2* and *hst* RNA; and preservation of *int-2* and *hst* open reading frames despite MMTV insertions. We did not observe insertions into the endogenous *Wnt-1* locus, an equally frequent target for MMTV insertions in tumors in normal BALB/c mice (24). This is the result expected in the presence of the *Wnt-1* transgene, supporting the idea that the frequency of observed integration sites reflects a growth advantage conferred by the insertions rather than a predisposition for MMTV to integrate into certain regions of the mouse genome.

The results reported also support earlier evidence for cooperation between *Wnt-1* and *int-2* obtained by infecting normal mice (20–22) and by crossing *Wnt-1* and *int-2* transgenic mice (25). Our results also extend the conclusions of those earlier studies by including another FGF gene, *hst*. It is noteworthy that the products of *Wnt-1* and FGF genes can collaborate in an entirely different experimental context: the induction of *Xenopus* mesoderm *in vitro* (26).

Coexpression of *int-2* and *hst* was observed in two tumors. A provirus was found in proximity to each gene in one of the tumors (126), suggesting that activation of both genes contributes to tumor formation. This would imply that *int-2* and *hst*, two members of the FGF family, are able to cooperate with each other, as well as with *Wnt-1*. Alternatively, it is possible that tumor 126 represents a mixture of independent neoplasms. Tumor 122 with a single *int-2*-activating provirus located 3′ of *int-2* also shows activation of *hst*. It is presently unclear why some insertions into this area (also see ref. 11) appear to strongly activate *hst* while others do not. This paradox could conceivably be explained by second proviruses, located outside the range of current probings, that activate *hst*. It is notable that we also found insertions on both sides of *int-2* in tumor 131 that produced transcripts of *int-2*, but not of *hst*. Unless *hst* RNA was present at levels precluding detection, it seems likely that this tumor sample represents a mixture of tumors, each with insertional mutations of *int-2*. Oligoclonality has previously been described in mouse mammary tumors (22, 28); the clonal populations within these tumors may grow independently or interdependently via paracrine communication.

Until recently, the major methods used to identify cooperating genetic events in cancer have depended on analysis of viral genomes that carry two oncogenes, cooperativity assays of known oncogenes in cell culture or in transgenic mice, or laborious methods to locate the involved loci in defined stages of human tumors (27). The method we have used here also appears to be useful for identifying cooperating protooncogenes. Using a similar strategy with murine leukemia virus, others have recovered both known and unidentified genes that cooperate in lymphomagenesis (5–7). In over half of our tumors with proviral insertions, the known targets for MMTV insertion mutations do not appear affected. The provirally tagged genes in these tumors could be a rich source of additional oncogenes implicated in mammary cancer.

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