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Transgenes Expressing the *Wnt-1* and *int-2* Proto-Oncogenes Cooperate during Mammary Carcinogenesis in Doubly Transgenic Mice

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The *Wnt-1* and *int-2* proto-oncogenes are transcriptionally activated by mouse mammary tumor virus insertion mutations in virus-induced tumors and encode secretory glycoproteins. To determine whether these two genes can cooperate during carcinogenesis, we have crossed two previously characterized lines of transgenic mice to obtain bitransgenic animals carrying both *Wnt-1* and *int-2* transgenes under the control of the mouse mammary tumor virus long terminal repeat. Mammary carcinomas appear earlier and with higher frequency in the bitransgenic animals, especially the males, than in either parental line. Nearly all bitransgenic males develop mammary neoplasms within 8 months of birth, whereas only 15% of *Wnt-1* transgenic males and none of the *int-2* transgenic males have tumors. In virgin bitransgenic females, tumors occur approximately 2 months earlier than in their *Wnt-1* transgenic siblings; *int-2* transgenic females rarely exhibit tumors. Preneoplastic glands from the bitransgenic animals of either sex demonstrate pronounced epithelial hyperplasia similar to that seen in *Wnt-1* transgenic virgin females and males, and both transgenes are expressed in the hyperplastic glands and mammary tumors. RNA from the *int-2* transgene is more abundant in mammary glands from bitransgenic animals than from *int-2* transgenic animals; the increase is associated with high levels of RNA specific for keratin genes 14 and 18, suggesting that *Wnt-1*-induced epithelial hyperplasia is responsible for the observed increase in expression of the *int-2* transgene.

The development of techniques for introducing genes into vertebrate germ lines has revolutionized the study of neoplasia by providing animal models in which potential oncogenes can be reproducibly expressed in target tissues (8, 19, 41). In this manner, using the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV) provirus to direct expression of transgenes in mouse epithelial tissues, the capacity of several oncogenes to induce mammary cancers has been forcefully documented (3, 27, 29, 31, 34, 35, 52, 55, 57, 58). Furthermore, by mating transgenic animals that express such transgenes in the same tissue (52), it has been possible to demonstrate cooperative oncogenic effects that may simulate the multistage evolution of naturally occurring tumors (13, 64).

This experimental strategy is particularly well suited to the study of those genes first implicated as oncogenes through insertional activation in MMTV-induced mammary cancers. Transgenes controlled by the MMTV LTR mimic the proviral insertion mutations encountered in virus-infected animals and offer exceptional opportunities to identify additional steps in a naturally occurring form of mammary neoplasia. To date, five such MMTV-activated genes have been identified: two members of the *Wnt* gene family, *Wnt-1* (formerly *int-1* [36, 38, 39]) and *Wnt-3* (48), which encode secretory glycoproteins (4, 42, 43); two members of the fibroblast growth factor family, *int-2* (10, 44) and *hst* (45); and the *int-3* gene (15), which encodes a transmembrane protein of unknown function (5a).

Before the advent of transgenic methods, the conviction that these genes had a causative role in mammary tumorigenesis depended upon two indirect lines of evidence: the observation that MMTV insertion mutations augmented mRNA production from the affected genes without disrupting protein-coding sequences (10, 11, 38, 61) and the finding that ectopic expression of those genes altered the morphology and growth potential of certain cultured cell lines (5, 17, 47). The case for *Wnt-1* as a mammary oncogene was greatly strengthened by the demonstration that both male and female transgenic mice programmed to express this gene in the mammary epithelium develop mammary hyperplasia that frequently progresses to malignancy (58). The incidence and kinetics of mammary tumorigenesis in these *Wnt-1* transgenic mice, however, suggest that additional pathogenic events are required. Transgenic mice that express the *int-2* gene in the mammary gland also develop mammary hyperplasia, but only in breeding females, and cancer develops infrequently and at a relatively late age (34, 40).

To ask whether the carcinogenic effects of *int-2* can be detected more readily in the presence of an activated *Wnt-1* gene, we have crossed previously described lines of *Wnt-1* and *int-2* transgenic mice to produce bitransgenic animals. The hypothesis that *Wnt-1* and *int-2* might be able to collaborate in mammary tumorigenesis was prompted by earlier observations that some tumors in MMTV-infected mice harbor insertion mutations of both *Wnt-1* and *int-2* (18,

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33, 46). In those studies, however, the timing of the two insertions could not be ascertained, and it was not clear whether the insertions had occurred in the same cell or whether the tumors were composed of two or more independently infected clones that might be stimulating each other by paracrine effects. In the genetic crosses reported here, bitransgenic animals show the pattern of mammary hyperplasia previously observed in *Wnt-1* transgenic animals and express both transgenes in the hyperplastic glands. Mammary carcinomas arise earlier and more frequently in both males and virgin females than in sibling mice bearing only the *Wnt-1* transgene. Thus, these studies establish an oncogenic role for *int-2* in collaboration with *Wnt-1*.

MATERIALS AND METHODS

Transgenic mice. The *Wnt-1* transgenic line used, 303 (58), was derived from a (C57BL × SJL) F_2 founder animal and has been maintained by breeding transgenic males with SJL females. The *int-2* transgenic line used, TG.NR (34), was derived from FVB/N animals and has been maintained by breeding transgenic females with nontransgenic FVB/N males. For the studies reported here, two *Wnt-1* transgenic males were mated to two *int-2* transgenic females and maintained in the UCSF Animal Care Facility. The F_1 progeny were screened for inheritance of transgenes as described below, and F_2 and F_3 generations were produced by brother-sister matings of *Wnt-1* transgenic males and *int-2* transgenic females. Over 40 animals in each of the relevant categories—males and females with the *Wnt-1* transgene, the *int-2* transgene, or both transgenes—were maintained under weekly surveillance for mammary tumors for at least 1 year or until tumors of 1 cm or more appeared.

Genotyping of progeny for transgenes. DNA was extracted from 0.5-cm tail sections of 3- to 4-week-old pups as detailed by Hogan et al. (21) by lysis in sodium dodecyl sulfate (SDS), digestion with proteinase K and RNase A, extraction in phenol-chloroform (1:1), and ethanol precipitation. DNA was digested with *Bam*HI, electrophoresed in a 1.0% agarose gel, transferred to nylon filters (Hybond N; Amersham), and analyzed for the presence of *Wnt-1* and *int-2* transgenes by hybridization with DNA probes radiolabeled with ^{32}P by nick translation (30). The *Wnt-1* probe was a 2.95-kb *Bam*HI-*Bgl*II fragment isolated from pM5.3-12 (58), and the *int-2* probe was a 1.69-kb *Bam*HI-*Hin*dIII fragment isolated from pKC3-9 (12) (see Fig. 1).

Examination of mammary glands by whole-mount and histopathological methods. Inguinal or thoracic mammary fat pads were removed under general anesthesia (2.5% tribromoethanol [Avertin] [21]). Each fat pad was spread in a Tissue Tek capsule, fixed in Tellesniczky's fixative, defatted in acetone, stained in iron hematoxylin, and analyzed in methyl salicylate under a 2.0× dissecting microscope as previously described (58). For histopathological examination, mammary tumors were excised, fixed in 10% formalin, embedded in paraffin, sectioned in 5-μm slices, and stained with hematoxylin and eosin.

Analysis of RNA from mammary glands and tumors. Whole-cell RNA was isolated from tissues by the acid-guanidine thiocyanide, phenol-chloroform method (6). RNA (5 μg per sample) was denatured with 1.2 M glyoxal, electrophoresed in 1% agarose gels, transferred to a Zeta Probe membrane (Bio-Rad), fixed by UV cross-linking, and hybridized with the indicated ^{32}P-labeled DNA probes at 65°C in hybridization buffer (7). Filters were washed at 70°C in 50 mM $NaHPO_4$ plus 1% SDS before autoradiography.

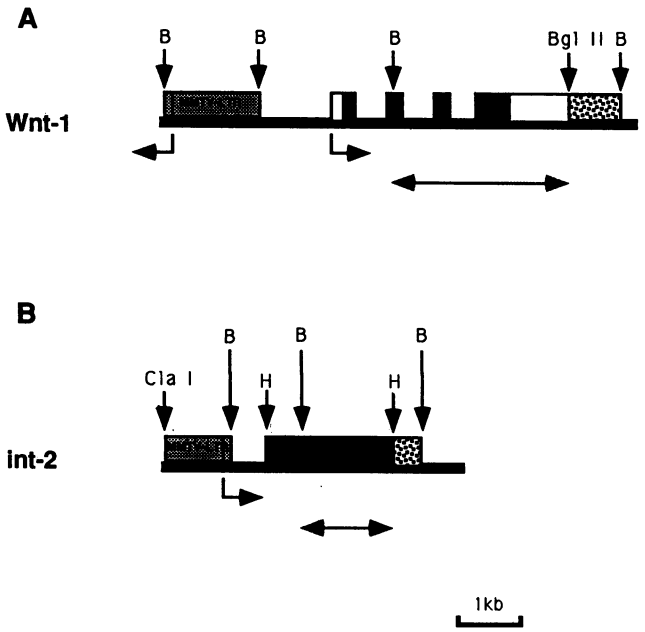

FIG. 1. Physical maps of the *Wnt-1* and *int-2* transgenes. (A) The *Wnt-1* transgene, including the known exons of mouse *Wnt-1* (14, 61) (coding regions are shaded), nearly all of the MMTV LTR (C3H strain) upstream of the first exon in the opposite transcriptional orientation, and a small piece of simian virus 40 DNA (stippled); for details, see reference 58. (B) The *int-2* transgene, with a truncated MMTV LTR (C3H strain) promoting transcription of mouse *int-2* cDNA and a polyadenylation site provided by simian virus 40 DNA (stippled). Restriction sites (vertical arrows) and radioactive probes (horizontal two-headed arrows) used in genotyping animals by Southern blotting are indicated for each transgene. B, *Bam*HI; H, *Hin*dIII.

The same filter was sequentially hybridized with *Wnt-1* cDNA (1.89-kb *Eco*RI-*Xba*I fragment from pBS KS *Wnt-1* [25]), mouse *int-2* cDNA (2.15-kb *Hin*dIII fragment from pKC3-9 [12]), mouse keratin 18 cDNA (1.5-kb *Sac*II-*Bam*HI fragment from pUC9B7 [51]), and mouse keratin 14 cDNA (3′ untranslated region; 450-bp *Hin*dIII-*Eco*RI fragment from K14-3′NC [49]) as probes. Between hybridizations, probes were stripped from filters by boiling for 1 min in 50 mM $NaHPO_4$ plus 1% SDS. To determine the number of copies of *Wnt-1* and *int-2* mRNAs per cell, autoradiographic signals were matched with results of parallel hybridizations performed with known amounts (0.2 to 2,000 pg) of *Wnt-1* and *int-2* RNAs synthesized in vitro. Five micrograms of cellular RNA was assumed to represent RNA from 1.5×10^5 cells.

RESULTS

Experimental design. To determine whether *Wnt-1* and *int-2* transgenes could cooperate during mammary tumorigenesis, we took advantage of existing transgenic mouse lines carrying the transgenes illustrated in Fig. 1. The *Wnt-1* transgene (Fig. 1A) mimics a common type of mutant allele from virus-induced tumors; the MMTV LTR is positioned 5′ of the first *Wnt-1* exon in a transcriptional orientation opposite that of the gene, so that it enhances transcription from the *Wnt-1* promoter (37, 38). The *int-2* transgene (Fig. 1B) contains part of the MMTV LTR upstream of the coding region of an *int-2* cDNA clone in the same orientation, and transcription is expected to be driven by the viral promoter.

Both of these transgenes are expressed in the mammary and salivary glands and in the male reproductive tract, but they produce different phenotypes. In the *Wnt-1* transgenic line used here (line 303), both male and virgin female animals display marked mammary hyperplasia, and one or more mammary carcinomas develop within the first year of life in some males and in virtually all females (58) (see Fig. 2). In the *int-2* transgenic line (TG.NR), persistent mammary hyperplasia is observed, but only in females that have undergone pregnancy; mammary cancers are uncommon and generally appear after 1 year of age; and prostatic hypertrophy with associated male infertility frequently occurs (34).

The inability of the hyperplastic mammary glands of line 303 females to deliver milk to newborns and the infertility of male TG.NR mice dictated the initial crosses of *Wnt-1* transgenic males with *int-2* transgenic females. Because the two transgenic lines had been created in different genetic backgrounds (see Materials and Methods), we attempted to minimize the effects of this difference by observing relatively large numbers of first-, second-, and third-generation progeny of crosses between line 303 males and line TG.NR females. Four cohorts of approximately 40 animals, males and females with one or both transgenes, were maintained for up to 1 year and carefully inspected at least once a week for the appearance of mammary tumors. To eliminate the timing and number of pregnancies as variables in the analysis, the female animals observed for the appearance of mammary pathology were not bred. As an additional control, a smaller number of nontransgenic females from these crosses were maintained for over 1 year; as anticipated, none of these animals developed mammary tumors (data not shown).

Time course of appearance of mammary tumors in progeny of *int-2* × *Wnt-1* transgenic animals. F_1, F_2, and F_3 progeny carrying a single *Wnt-1* or *int-2* transgene displayed the mammary gland characteristics previously described for the respective parental lines, suggesting that the effects of genetic background were minimal with respect to the traits of major interest. Among virgin females, about 60% of the *Wnt-1* transgenic animals developed mammary carcinomas by 8 months of age, whereas none of the corresponding *int-2* transgenic animals exhibited tumors; likewise, none of the *int-2* transgenic males developed mammary tumors, but about 10% of the *Wnt-1* transgenic males did so by 8 months of age (Fig. 2). As shown below, the *Wnt-1* transgenic males and virgin females both developed the type of mammary hyperplasia previously described for line 303, whereas hyperplasia was not observed in the *int-2* transgenic animals unless the females became pregnant, as reported for line TG.NR (34). Enlarged prostate glands were encountered in *int-2* transgenic males derived from our cross, although (for unknown reasons) the onset was delayed and the hypertrophy less marked in bitransgenic males (data not shown).

Mammary tumors appeared earlier in both male and female bitransgenic animals; in the males, tumors also occurred more frequently than in their monotransgenic littermates. Approximately half of the bitransgenic virgin females developed tumors before the age of 4 months, by which time fewer than 30% of the *Wnt-1* transgenic and none of the *int-2* transgenic virgin females had palpable tumors (Fig. 2A). Furthermore, about 90% of the bitransgenic virgin females exhibited tumors by 8 months of age, at which time 40% of the *Wnt-1* transgenic virgin females still appeared to be tumor free. Thus, the *int-2* transgene, which did not induce mammary tumors on its own in the course of this experiment, advanced the appearance of tumors by 1 to 2 months in virgin females carrying the *Wnt-1* transgene.

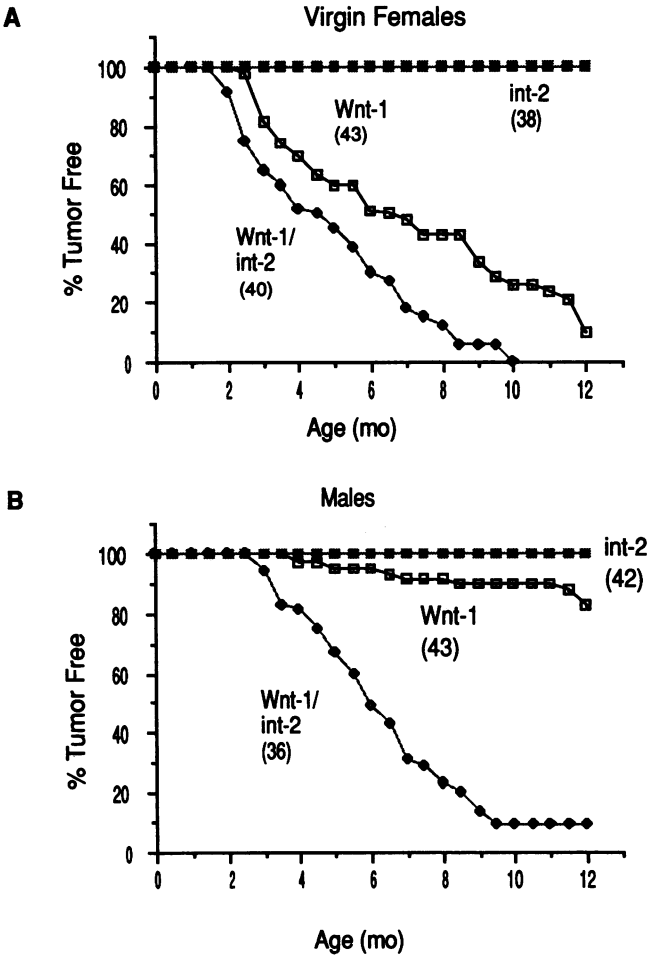

FIG. 2. Incidence of mammary tumors in bitransgenic, *Wnt-1* transgenic, and *int-2* transgenic progeny. The percentage of animals in each cohort remaining free of palpable mammary tumors was plotted at biweekly intervals as a function of age for virgin females and males. The numbers of animals in each group are indicated in parentheses.

This cooperative effect was more striking in the transgenic males (Fig. 2B). Over 70% of the bitransgenic males developed palpable mammary tumors by 7 months of age, at a time when fewer than 10% of *Wnt-1* transgenic males showed tumors. Moreover, by 1 year of age, only about 15% of *Wnt-1* transgenic males had developed mammary tumors, indicating that the presence of both transgenes dramatically increased the proportion of male animals developing tumors and also shortened the average latent period.

Histopathology of tumors. To ascertain whether the tumors observed in the bitransgenic animals were morphologically distinct from those arising in the *Wnt-1* transgenic animals, histological examination was performed on tumor specimens from six virgin female and two male bitransgenic animals. In all cases, the tumors were found to be adenocarcinomas closely resembling those previously described for *Wnt-1* transgenic mice (58) and for MMTV-infected animals (32). Representative sections from tumors arising in a *Wnt-1* transgenic female (Fig. 3A) and a bitransgenic male (Fig. 3B) display similar abnormalities of cellular morphology, occa-

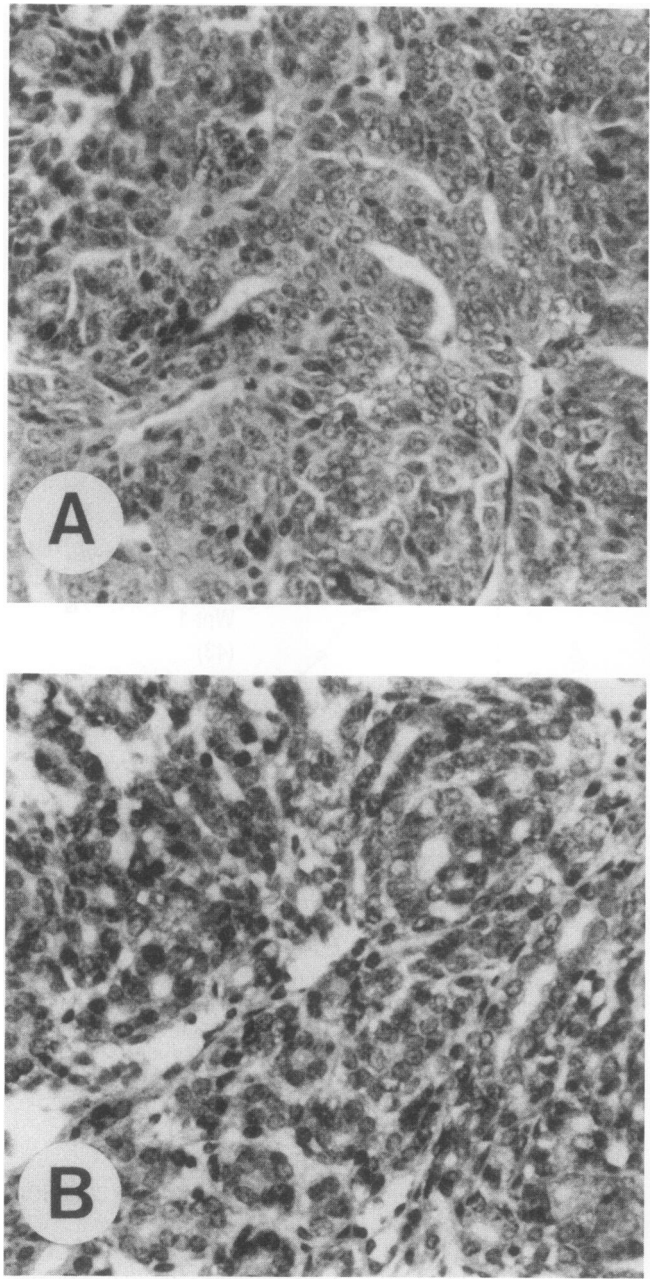

FIG. 3. Histopathology of mammary tumors from transgenic animals. Tumor samples, prepared as described in Materials and Methods, were photographed at ×200 magnification. (A) Primary tumor from a mammary gland of a 5.5-month-old, *Wnt-1* transgenic virgin female; (B) pulmonary metastasis from a 7.0-month-old, bitransgenic male with a palpable tumor in a mammary gland.

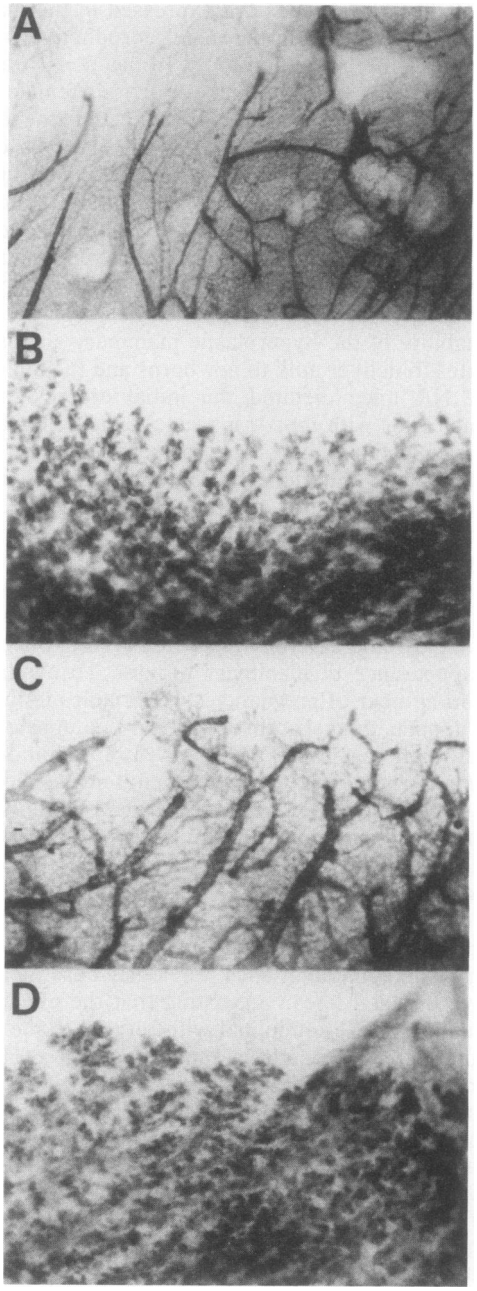

FIG. 4. Whole mounts of preneoplastic mammary glands. Fat pads were prepared as described in Materials and Methods from 2.0-month-old nontransgenic (A), *Wnt-1* transgenic (B), *int-2* transgenic (C), and bitransgenic (D) virgin animals. Panels A and C illustrate ductal patterns and panels B and D show lobulo-alveolar patterns, as summarized in Table 1. Magnification, ×19.

sional mitoses, and well-formed glandular lumens. Tumors from lactating females exhibited abundant secretory activity (not shown). Although we sacrificed tumor-bearing bitransgenic animals when the tumors were relatively small (generally less than 2.0 cm in diameter), pulmonary metastases were grossly apparent in tissue sections from three of the six females and in both males; an example of such a pulmonary lesion is depicted in Fig. 3B.

Preneoplastic mammary glands. Mammary glands from several progeny of the transgenic cross were prepared as whole mounts and stained to assess the developmental status (Fig. 4 and Table 1). As anticipated from our previous descriptions of lines 303 and TG.NR, *Wnt-1* transgenic animals, including virgin females and males, showed abundant mammary hyperplasia (e.g., Fig. 4B), whereas glands from *int-2* transgenic males and virgin females contained only smooth ducts, with little or no alveolar development buds, resembling glands from nontransgenic virgin animals (Fig. 4A and C). Bitransgenic virgin females and males

TABLE 1. Mammary gland hyperplasia in *Wnt-1* and bitransgenic animals

Animals	Transgene(s)		Mammary gland status[a]		
	Wnt-1	*int-2*	Duct only	Alveolar bud	Lobulo-alveolar
Virgin females	−	−	10	0	0
	+	−	0	4	4
	−	+	7	0	0
	+	+	0	6	7
Males	−	−	6	0	0
	+	−	0	3	9
	−	+	3	0	0
	+	+	0	2	4

[a] Whole mounts of mammary fat pads from male and virgin female animals with the indicated genotypes were scored for developmental status as described in the text. Ductal patterns (Fig. 4A and C) are normal for nontransgenic males and virgin females; alveolar budding and lobulo-alveolar patterns (Fig. 4B and D) indicate proliferation of the mammary epithelium.

developed mammary glands indistinguishable from those observed in *Wnt-1* transgenic animals (Fig. 4D). As summarized in Table 1, all of the tested mammary glands from animals carrying the *Wnt-1* transgene showed moderate to marked glandular development, manifested by alveolar buds or by the more advanced lobular alveolar outgrowths illustrated in Fig. 4B and D. Fat pads from other animals, with or without the *int-2* transgene, displayed an unstimulated ductal pattern.

Expression of transgenes in mammary glands and tumors. The findings presented to this point suggest that the *int-2* transgene enhances the previously described oncogenic potential of the *Wnt-1* transgene in virgin female and male animals, but examination of preneoplastic and tumor tissue did not reveal how the *int-2* transgene might contribute to pathogenesis. To begin to dissect the molecular mechanisms of cooperation between these transgenes, we used the Northern blot (RNA blot) procedure to analyze RNA from mammary glands and tumors from male and virgin female animals with one or both transgenes. Our initial concerns were to ascertain that each transgene was expressed in mammary tissues and to ask whether either of the transgenes might modulate expression of the other.

Since the size and cellular composition of the mammary glands were influenced by the presence of transgenes (Fig. 4), we probed RNA samples sequentially for each of the transgene mRNAs and then for several cellular mRNAs, including those encoding cDNAs specific for mouse keratins 14 and 18. The keratin 14 mRNA is known to be produced at especially high levels in proliferating mammary cells, including stem cells and myoepithelium, whereas keratin 18 mRNA is abundant in all mammary epithelial cells (53, 56). By normalizing our results to the expression of these keratin genes, we hoped to distinguish between differences in levels of transgenic RNA that could be accounted for by differences in the abundance of the various mammary cell types and those that might be due to augmented expression in individual cells.

Representative analyses of nontransgenic, *Wnt-1* transgenic, *int-2* transgenic, and bitransgenic mammary glands and of *Wnt-1* transgenic and bitransgenic mammary tumors are presented in Fig. 5 (virgin females) and Fig. 6 (males). As expected, abundant *Wnt-1* transgenic mRNA, up to 1,000 copies per cell, was detected in the hyperplastic mammary

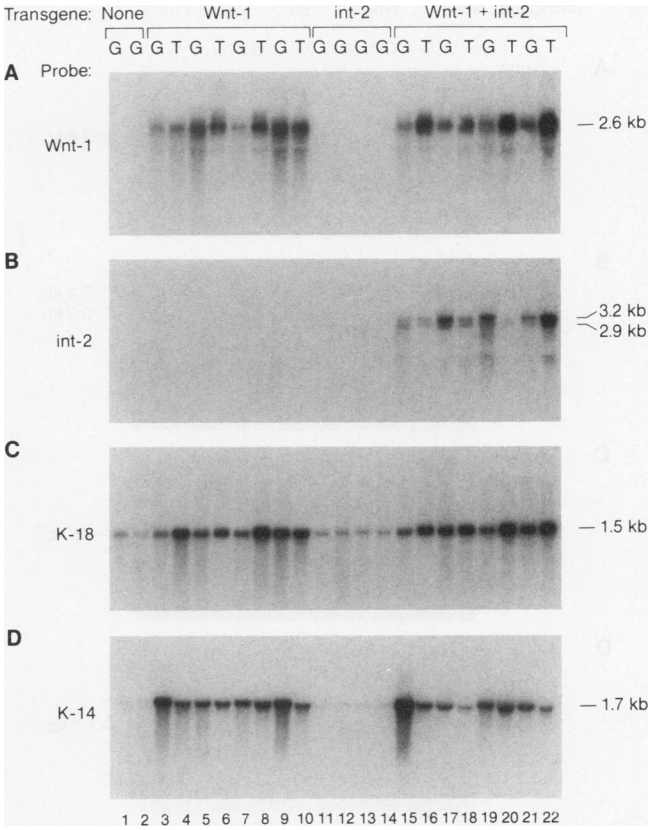

FIG. 5. Northern blot analysis of RNA from mammary glands and tumors of transgenic females. Each lane contains 5 μg of whole-cell RNA from preneoplastic mammary glands (G) or mammary tumors (T) from virgin females carrying no, *Wnt-1*, *int-2*, or both transgenes. After gel electrophoresis and transfer to filters, the samples were tested by sequential hybridization with ^{32}P-labeled probes for *Wnt-1* (A), *int-2* (B), keratin 18 (C), and keratin 14 (D), as detailed in Materials and Methods. The positions of the RNA species of interest, deduced from the positions of rRNAs, are indicated at the right.

glands and mammary tumors from *Wnt-1* transgenic and bitransgenic animals but not in samples from the nontransgenic or *int-2* transgenic animals. In general, there was a strong correlation between the levels of *Wnt-1* mRNA and levels of keratin (especially keratin 18) mRNA in samples from animals carrying the *Wnt-1* transgene; this relationship was especially evident in the few male mammary gland samples that showed very low levels of *Wnt-1* mRNA, presumably because of a paucity of mammary cells (e.g., lanes 5, 11, and 13 in Fig. 6).

Small amounts of the 3.2- and 2.9-kb *int-2* transgenic mRNAs were observed in undeveloped mammary glands from virgin female and male *int-2* transgenic mice, consistent with a previous report (34); these *int-2* transcripts were not detected in control or *Wnt-1* transgenic materials. In contrast, moderate to abundant amounts of *int-2* transgenic RNA were found in most of the bitransgenic glands and tumors (Fig. 5, lanes 15 to 22; Fig. 6, lanes 11 to 17). Again, the highest levels (up to 1,000 copies per cell in tumors from females or males) were associated with abundant keratin mRNAs, whereas small amounts of keratin mRNA were detected in bitransgenic samples with the lowest levels of *int-2* mRNA (less than 5 copies per cell) in male mammary glands (Fig. 6, lanes 11, 13, and 15).

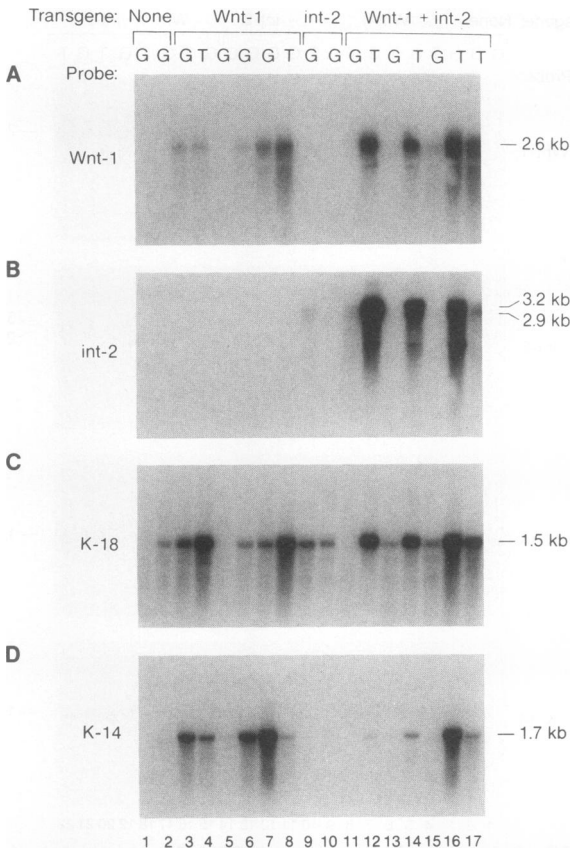

FIG. 6. Northern blot analysis of RNA from mammary glands and tumors of transgenic males. RNA from mammary glands (G) or tumors (T) from males with the indicated genotypes was analyzed as described in the legend to Fig. 5.

The apparent correlation between levels of keratin and *int-2* transgenic RNAs in *int-2* and bitransgenic animals suggests that much of the increase in *int-2* RNA in the bitransgenic animals can be attributed to a relative increase in the number of mammary epithelial or myoepithelial cells, resulting from the hyperplasia stimulated by the *Wnt-1* transgene. However, since we do not know which cell types express the transgenes or whether expression is uniform within any subpopulation of cells, we cannot exclude the possibility that *Wnt-1* specifically induces higher levels of the *int-2* transgene in some cells. Other ways in which the two transgenes might cooperate are considered in the Discussion.

DISCUSSION

We have shown that mice inheriting *Wnt-1* and *int-2* transgenes programmed for expression in the mammary gland develop mammary carcinomas earlier and more frequently than mice inheriting either of these transgenes individually. Our findings extend the existing evidence for a role for *int-2* in mammary oncogenesis and confirm previous inferences from studies of MMTV-infected, nontransgenic mice (18, 33, 46) that *Wnt-1* and *int-2* can cooperate during mammary tumorigenesis. We have subsequently obtained additional evidence for the collaboration of these two genes: infection of *Wnt-1* transgenic mice with MMTV accelerates mammary carcinogenesis, and many of the tumors contain proviral insertion mutations of *int-2* (50a).

Cancer is generally considered to result from an accumulation of mutations affecting proto-oncogenes and tumor suppressor genes (2, 64). A wide variety of evidence in support of this view has emerged from the study of DNA and RNA tumor viruses carrying multiple oncogenes (22), from transformation of cultured cells with two or more oncogenes (26, 50), from enumeration of genetic lesions in human and animal cancers (13), and from studies of tumor progression (28). Transgenic mice provide especially tractable models for evaluating the contributions made by multiple genetic components to a complex pathogenic process; this is particularly true when the target cell is difficult to grow and analyze in cell culture. Combinations of genes that can accelerate tumorigenesis in mammary and hematopoietic lineages have been documented by crossing transgenic animals carrying different activated oncogenes (52, 62; this report) and by infecting transgenic animals with retroviruses that cause insertion mutations (20, 50a, 59, 60). The latter method has the additional virtues of identifying unexpected combinations of known genes and facilitating the discovery of novel genes (20, 60).

In many instances of demonstrated cooperation between pairs of oncogenes, one gene encodes a protein that acts in the nucleus (e.g., a putative transcriptional regulator, such as a Myc protein) and the other encodes a protein that acts in the cytoplasm or at the plasma membrane (e.g., a component of the signal transduction pathway, such as a Ras protein) (22, 63). There are, however, several exceptions to this formulation—for example, the cooperation between two genes, c-*myc* and *bmi-1*, that both encode nuclear proteins predicted to be transcription factors (20, 60). In the collaboration between *Wnt-1* and *int-2* documented here, both genes encode secretory glycoproteins. *int-2* protein, which is closely related to fibroblast growth factors (9, 54), is presumed to interact with one of the known fibroblast growth factor receptors (16, 23, 24) or a very similar molecule and thereby to induce protein-tyrosine phosphorylation. It seems intuitively likely that genes with additive effects in tumorigenesis will affect different pathways governing cell proliferation or different steps in a common pathway, regardless of the site(s) at which their products act. Since receptors for *Wnt* proteins have not yet been identified, it is not possible to say whether *Wnt-1* and *int-2* proteins activate different signaling pathways. Moreover, we cannot exclude the unlikely possibility that the relevant product of the *int-2* transgene is the nuclear protein initiated from a CUG codon upstream of the major initiation site (1), since the upstream initiation site is included in the transgene.

We have attempted to determine whether *Wnt-1* and *int-2* transgenes cooperate by inducing the expression either of one another or of endogenous *Wnt-1* or *int-2* proto-oncogenes in the target tissue. Within the limits of the assays used in Fig. 5 and 6, it appears that the *int-2* transgene has no significant effect upon the production of transgenic or endogenous *Wnt-1* mRNA. Mammary tissues from bitransgenic animals frequently displayed much higher levels of *int-2* RNA than were found in animals inheriting only the *int-2* transgene, but tests with probes for keratin RNAs (panels C and D in Fig. 5 and 6) and examination of mammary fat pads (Fig. 4 and Table 1) suggested that the larger amounts of *int-2* RNA are proportional to the *Wnt-1*-induced increases in the number of mammary epithelial cells. Nevertheless, significant effects upon expression of *Wnt-1* or *int-2* might have

occurred in subsets of cells that would not have been detected with the methods used here.

We have also considered the possibility that *Wnt-1* and *int-2* cooperate by inducing receptors for each other's gene products. Although it is, of course, not yet possible to test for regulation of the *Wnt-1* receptor, we have made a preliminary effort to measure the amounts of fibroblast growth factor receptor RNAs in the samples analyzed in Fig. 5 and 6. Thus far, similar levels of RNAs have been observed in all samples, by using probes for fibroblast growth factor receptors encoded by three different genes (43a).

Although our results clearly demonstrate that both the *Wnt-1* and *int-2* transgenes can contribute to mammary carcinogenesis, the kinetics of tumor induction in bitransgenic animals suggests that activation of these proto-oncogenes is not sufficient for development of a carcinoma. Thus, mammary cancers do not appear in these mice until several months after birth (Fig. 2), and fewer than 30% of the animals harbor tumors in more than 1 of the 10 mammary glands at the time the first tumor is detected (25a). In view of these findings, it appears likely that additional, as yet unidentified genetic or epigenetic events are needed for full expression of malignancy. The bitransgenic mice described in the present report may provide a useful model in which to identify these additional events in mammary carcinogenesis.

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