Insertion Mutation of the *int-1* and *int-2* Loci by Mouse Mammary Tumor Virus in Premalignant and Malignant Neoplasms from the GR Mouse Strain

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Mouse mammary tumor virus (MMTV)-induced mammary adenocarcinomas can develop from several different premalignant precursors common in GR mice. Insertion mutagenesis of the mammary proto-oncogenes *int-1* and *int-2* was studied in this multistep system by analyzing samples from various stages of neoplastic development for novel *int-1* and *int-2* restriction fragments generated by MMTV provirus integration. *int-1* and *int-2* insertion mutations were observed in both premalignant lesions and malignant tumors. Some of the tumors with insertion mutations were experimentally derived from insertion mutation-free premalignant precursors. Each class of neoplasm examined had a characteristic frequency of *int-1* and *int-2* insertion mutations; however, no correspondence was observed between neoplasm morphology and mutation of either gene. These results indicate that insertion mutation of the *int-1* and *int-2* loci by MMTV provirus can be involved in the earliest identifiable stages of neoplastic development as well as during progression of premalignant lesions to tumors. Insertion mutation of *int-1* and *int-2* is therefore not stage specific in this system.

Tumorigenesis is a progressive process requiring a series of somatic changes before development of the malignant phenotype (18, 37). In some tumor systems, the progressive nature of tumor development is reflected in the occurrence of morphologically defined premalignant precursors (15, 16). The mouse mammary tumor system is a prototypic example (25). Mouse mammary adenocarcinomas can develop from a variety of different premalignant precursors, including two lesions studied in this report: hyperplastic alveolar nodules (HANs) and plaques (see Fig. 1).

HANs are focal proliferations of lobuloalveolar epithelium, observed most frequently in the unstimulated mammary glands of multiparous females (21). These lesions contain immortal cells that can be propagated indefinitely as hyperplastic outgrowths (HOGs) by serial transplantation in gland-free mammary fat pads of virgin syngeneic hosts (6, 21). HOGs will not overgrow normal mammary gland (12) or grow outside the mammary fat pad (11). HANs and HOGs retain a high degree of differentiation, morphologically resembling normal hormone-stimulated, prelactating mammary glands. In comparison, plaques are less differentiated, having a closer morphological resemblance to mammary adenocarcinomas (13). Plaques require endocrine stimulation for growth and arise during pregnancy. They regress at parturition, but reappear during subsequent pregnancies. Mammary adenocarcinomas are postulated to arise from HANs, HOGs, and plaques by progressive clonal selection of variants with increased growth autonomy (2, 14).

Mouse mammary tumor virus (MMTV) is one of the principal etiologic agents in the development of HANs, plaques, and mammary adenocarcinomas. A common feature of all three lesions is the presence of clonally integrated exogenous MMTV proviruses (1-4, 9, 10). Southern blot analysis of these lesions with MMTV probes reveals novel host-virus junction restriction fragments present in clonal

(although not necessarily monoclonal) stoichiometries. A hypothesis for the role of these exogenous proviruses in mammary tumorigenesis was suggested by Nusse and Varms (28). In mammary tumors from the C3H strain, MMTV proviruses were frequently found integrated within a 20-kilobase (kb) region encompassing the mammary proto-oncogene *int-1*, leading to transcription of the otherwise transcriptionally inactive gene (27).

We tested the insertion mutagenesis model in the GR mouse strain, which has a high frequency of MMTV-induced HANs, plaques, and mammary adenocarcinomas (35). The GR mouse strain has five endogenous MMTV proviruses (19). One of these proviruses, *Mtv-2*, is transcriptionally active and present at increased copy number in GR neoplasms (9, 10). Host DNA flanking a single clonally integrated, newly acquired MMTV (GR) provirus observed in a GR tumor was cloned, and probes for this region of the mouse chromosome were developed. Exogenous provirus was detected at this locus in independent tumor samples by Southern blot analysis with these probes. Further characterization revealed that this region was allelic with the *int-2* locus cloned and characterized by Dickson, Peters, and co-workers from the BALB/cfC3H strain (8, 30). The *int-2* locus is similar in size to the *int-1* locus and encompasses a mammary proto-oncogene related to acidic and basic fibroblast growth factors (C. Dickson and G. Peters, Letter, Nature [London] 326:833, 1987) and the *hst/K-fgf* oncogene (5, 38). *int-2* expression was shown to be *cis*-activated by MMTV provirus insertion in a manner analogous to *int-1* activation (8, 27).

In this report, we present our *int-2* cloning experiments and the results of a comparative study of GR mammary lesions from different stages of neoplastic development by using probes from the C3H *int-1* and GR *int-2* loci. The goal of this survey was to examine as many types of lesions as possible from a single mouse strain to determine whether correlations exist between the alternate pathways of mam-

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mary pathogenesis and insertion mutagenesis of *int*-class genes.

MATERIALS AND METHODS

Mice. GR mice (26) were obtained from the Netherlands Cancer Institute, Amsterdam. In this report, sporadic mammary tumors of undetermined pathogenesis found in non-pregnant, nonlactating female breeders from our GR colony are called spontaneous tumors (Fig. 1). No measurements of tumor size were performed between pregnancies to determine whether tumors were hormone responsive. Plaques and HANs were identified by surgically exposing the mammary glands of pregnant and nonpregnant, nonlactating mice, respectively, and looking for abnormal growth under a dissecting microscope. HANs appeared as small (<1-mm³) focal lesions of lobuloalveolar development within unstimulated ductal mammary gland (Fig. 1). Plaques were larger, disk-shaped lesions (with a long axis of ca. 5 to 10 mm) observed in the mammary glands of midpregnant mice (Fig. 1).

Transplantation. Serial transplantation lines from HANs found in both virgin and multiparous females were established by standard techniques (6, 21). The developing mammary glands were surgically removed from the no. 4 mammary fat pads of 3-week-old virgin GR females, and the resulting gland-cleared fat pads were used as transplant recipients. At this age, <20% of the fat pad contains glandular tissue. HAN or HOG tissue was implanted into the cleared fat pads at the time of surgery. These transplants were allowed to grow for 3 to 6 months before retransplantation and/or collection of tissues for analysis.

Histology. Mammary tissues for histological analysis were fixed *in situ* as mammary whole mounts by immersion of mouse pelts in 70% ethanol-acetic acid-formaldehyde (20:1:1) for at least 24 h. Fixed mammary glands were peeled from the pelts, delipidated with acetone, and stained with hematoxylin (21). The ductal, lobuloalveolar, and neoplastic areas of the mammary glands could be clearly distinguished after this processing. Areas for histological analysis were dissected, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

DNA cloning. Experimental details of the genomic library construction and screening are presented elsewhere (24). Briefly, a genomic library was constructed in bacteriophage lambda vector λ L47 by using *Mbo*I partial digestion fragments from tumor DNA. The tumor DNA was obtained from spontaneous mammary tumor sample GRTu7 bearing a single clonally integrated exogenous provirus (Fig. 2A). The library was plated on *Escherichia coli* KH802 and screened by plaque hybridization with ³²P-labeled probe CP4.2 from the *gag-pol* region of MMTV(C3H) (see below). Two clones of the exogenous GRTu7 provirus were identified, λ GRT6 and λ GRT39 (Fig. 2B). Viral and flanking DNA probes were subcloned as described previously (24).

Probes. pBR322 clones of three of the four internal *Pst*I restriction fragments from unintegrated exogenous MMTV(C3H) proviral DNA were provided by Majors and Varmus (20). The *gal-pol*, *env*, and long terminal repeat (LTR) clones were subcloned into pUC12 and named pCP4.2, pCP1.8, and pCP1.4, respectively. pCP/X0.4 is a pUC12 subclone of the 5' 0.4-kb *Pst*I-*Xba*I fragment from the pCP4.2 insert. pDM5 is a pUC12 subclone of the 1.3-kb *Pst*I-*Pvu*II fragment from λ GRT39 containing the MMTV(GR) 5' LTR. *int-1* probe C was provided by Nusse and Varmus (28). *int-2* probes p39A and p6E are pUC12

subclones of lambda clones λ GRT39 and λ GRT6, respectively, that contain mouse DNA flanking the GRTu7 exogenous provirus (Fig. 3). p39A has a 0.6-kb *Sst*I-*Pst*I insert, with the *Pst*I site occurring 8 nucleotides inside the λ GRT39 provirus 5' LTR. p6E has a 0.6-kb *Bam*HI-*Eco*RI insert.

Southern blot analysis. DNA samples were prepared and analyzed as previously described (24). Filters for rehybridization with a different probe were stored damp at -20°C, and old probe was removed by soaking the filters in distilled water at 70°C for 10 min. Probe removal was verified by autoradiography before rehybridization.

Most novel *int* gene fragments detected were reprobbed with various MMTV probes to verify that the fragments were due to MMTV provirus insertion. If the cause of a putative MMTV insertion mutation could not be verified in this manner because of comigrating endogenous proviral fragments, the sample in question was analyzed with additional restriction enzymes. The orientation of proviruses with respect to the *int-1* and *int-2* genes was determined by hybridizations with probes CP/X0.4 and CP1.8 specific for the *gag* and *env* regions, respectively, of MMTV. All samples with novel *int* gene fragments that electrophoresed at a higher molecular weight than germ line fragments were analyzed with other enzymes producing smaller fragments before being scored as bearing bona fide insertion mutations (to avoid scoring potential partial digestion fragments as insertion mutations). Overlapping restriction fragments were used in the survey to minimize the possibility of missing novel *int-1* or *int-2* locus restriction fragments because of chance comigration of these fragments with normal undisrupted fragments from the uninvolved homologs and normal stroma.

Restriction mapping. Restriction fragment sizes were determined by using the SIZER program (IntelliGenetics, Inc., Mountain View, Calif.) (34). Restriction fragment sizes of genomic fragments on Southern blots were determined by adding 50 ng each of High Molecular Weight DNA Markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and *Bst*EII-cleaved lambda DNA (New England BioLabs, Inc., Beverly, Mass.) to the loading buffer of genomic digests and reprobbed the filters with radiolabeled lambda DNA after hybridizations with *int* or MMTV probes were complete. Filters were marked with spots of India ink mixed with [¹⁴C]DNA (Amersham Corp., Arlington Heights, Ill.) to allow precise alignment of multiple films from reprobbed the same filter. This method gave more precise results than the standard technique of using a parallel marker lane because the problem of lane-to-lane variation was eliminated by the presence of an internal standard in each sample.

RESULTS

Isolation of the *int-2* locus from a GR mammary tumor with a single new exogenous provirus. A GR mammary tumor, GRTu7, was identified that had a single newly acquired MMTV(GR) provirus (Fig. 2A). This provirus and mouse flanking DNA were cloned in bacteriophage λ L47 (Fig. 2B). λ GRT39 contained the entire exogenous provirus with both 5' and 3' host flanking DNA and is described in detail elsewhere (24). λ GRT6 was truncated in the *pol* gene and contained 3' host flanking DNA encompassing the majority of the *int-2* gene (23). Two single-copy flanking DNA probes, probes 39A and 6E (Fig. 3), were subcloned and used to identify MMTV insertion mutations at this locus in independent tumor samples.

Survey of GR neoplasms from different stages of neoplastic

FIG. 1. Histology of premalignant and malignant lesions from the GR mouse strain. Transverse 4- μ m sections through whole mounts of no. 3 (A, B, F, and G) and no. 4 (C, D, E, H, I, and J) mammary fat pads, and a 4- μ m section through a typical spontaneous mammary tumor (K) are shown. (A and F) Normal unstimulated mammary gland from a virgin female. (B and G), HAN in an unstimulated mammary gland from a virgin female. (C and H) 168B HOG transplant. (D and I) Type A mammary adenocarcinoma in a 168B HOG transplant. (E and J) Imperfect plaque from a midpregnant female. (K) Type B mammary adenocarcinoma from a nonpregnant, nonlactating female GR breeder. Size markers for panels A to E (low magnification) and panels F to K (high magnification) are shown in panels D and K, respectively. The sections in panels A to E are magnified $\times 25$, and the sections in panels F to K are magnified $\times 180$.

FIG. 2. Cloning of the host DNA flanking the exogenous MMTV provirus from a GR tumor sample with a single new provirus. (A) Southern blot comparison of normal control GR spleen (lane 1) and GR mammary tumor GRTu7 (lane 2). Sample DNAs were digested with *Pst*I and hybridized with 32 P-labeled MMTV(C3H) LTR probe CP1.4. The novel 3' host-virus junction fragment from the single clonally integrated exogenous provirus present in GRTu7 is indicated by an arrow. This exogenous provirus and 5' and 3' host flanking DNA were cloned in bacteriophage lambda vector λ L47. (B) Restriction map of clones λ GRT6 and λ GRT39. Two clones of the GRTu7 exogenous provirus were recovered. λ GRT6 was truncated in the *pol* gene and extended through most of the *int-2* gene. λ GRT39 contained the entire provirus and both 5' and 3' host flanking DNA (24). The restriction map of the mutated *int-2* locus included in clones λ GRT6 and λ GRT39 is shown. Only enzymes used in this study to screen the *int-1* and *int-2* loci for insertion mutations are included. The locations of the *int-2* exons are from Moore et al. (23). The GRTu7 mutation is also shown in Fig. 3 (mutation T7). Abbreviations: Bc, *Bcl*I; B, *Bgl*II; E, *Eco*RI; K, *Kpn*I; X, *Xba*I.

development for insertion mutations of the *int-1* and *int-2* mammary proto-oncogenes. The genomic regions recognized by *int-1* probe C (28) and *int-2* probes 39A and 6E (Fig. 3) were analyzed with numerous restriction enzymes to search for enzymes that yielded large fragments. From these data, a strategy was developed to survey the *int-1* and *int-2* loci for insertion mutations (Fig. 3). To survey the *int-2* locus, *Xba*I and *Kpn*I digests were probed with probe 39A, and *Bcl*I digests were probed with probe 6E. This strategy allowed analysis of approximately 55 kb of the mouse genome encompassing the *int-2* gene. A similar strategy was developed for *int-1*. *Xba*I, *Kpn*I, *Bgl*II, and *Eco*RI digests were probed with probe C, allowing analysis of approximately 49 kb of the mouse genome encompassing the *int-1* gene.

A total of 72 GR neoplasms from different stages of neoplastic development (Fig. 1) were analyzed for MMTV insertion mutations of *int-1* and *int-2*. Insertion mutations were observed in 29 samples: 12 mutations at the *int-1* locus and 19 mutations at the *int-2* locus. Two spontaneous tumors had mutations at both loci. A summary of the survey data is

presented in Table 1. All of the HOG-derived tumors listed were from transplants of HOG lines free of *int-1* and *int-2* insertion mutations. The integration sites and orientations of the MMTV proviruses observed at *int-1* and *int-2* loci in spontaneous tumors, plaques, and HOG-derived tumors are shown in Fig. 3. In addition, a notable insertion mutation that occurred in an HAN is shown in detail in Fig. 5.

Insertion mutations of *int-1* and *int-2* during progression of premalignant HOGs to tumors. As previously observed, exogenous MMTV proviral restriction patterns for each HOG line were unique and served as markers for clonal cell populations (2). The progenitor HOG restriction patterns were conserved in the tumors that arose in HOG transplants, indicating that most, if not all, of the epithelial cells in HOG-derived tumors arose by clonal progression of the major HOG cell population to malignancy, rather than by emergence of tumors from cryptic cell populations in the HOGs. In addition, the HOG-derived tumors had unique host-virus junction fragments from new proviral copies integrated during progression of HOGs to tumors. Six of

FIG. 3. Localization and orientation of MMTV provirus insertions in the vicinity of the *int-1* and *int-2* genes. Regions flanking the *int-1* and *int-2* genes were assayed for MMTV insertion mutations by Southern blot analysis with the restriction fragments and probes diagrammed. The normal sizes and locations of the restriction fragments analyzed are shown below the map of each locus. Approximately 49 kb encompassing the *int-1* gene and 55 kb encompassing the *int-2* gene were analyzed. The locations of the *int-1* exons are from van Ooyen et al. (36), and the locations of the *int-2* exons are from Moore et al. (23). Insertion mutations were indicated by the presence of new restriction fragments not present in normal control DNA that also hybridized to MMTV probes. The locations and orientations of the proviruses involved were determined as discussed in the text. A vertical line marks the location of each provirus, and an arrow points 5' to 3'. Sample numbers are given next to the appropriate arrows. T7 is referred to elsewhere in the text as GRTu7. The insertion mutation discovered in HAN 350 is diagrammed in Fig. 5B and is not listed here. Abbreviations: T, spontaneous tumor; HT, HOG-derived tumor; P, plaque.

these newly integrated proviruses were found at the *int-1* and *int-2* loci (Table 1; Fig. 3).

Therefore, *int-1* and *int-2* insertion mutations were occasionally seen during the progression of clonal, immortalized HOG cells to malignant tumor cells with full growth autonomy. An example is illustrated in Fig. 4. A comparison of the

int-2 (Fig. 4A) and MMTV (Fig. 4B) restriction patterns of DNA from the 168B GR HOG transplantation line and tumor HT4 that arose in one of the transplants is shown. The 168B HOG line-specific host-virus junction fragments are marked (open arrows), as is the new junction fragment from an *int-2* MMTV insertion mutation (solid arrow). The integration site and orientation of this provirus in tumor HT4 are shown in Fig. 3. A whole mount of a 168B transplant is also shown (Fig. 4C), demonstrating the gross morphology characteristic of all the GR HOG transplantation lines. The hyperplastic morphology of this premalignant intermediate-stage lesion resembles the normal hyperplasia of pregnancy.

Insertion mutation of *int-2* during development of plaques. Several GR plaques were analyzed histologically, and all lacked a clearly defined border and central cortex (Fig. 1). This placed them in the imperfect-plaque subcategory described by Foulds (13). Insertion mutations of the *int-2* locus were observed in approximately half of the plaques examined by Southern blot analysis (Table 1; Fig. 3). In contrast to the results of the *int-2* analyses, no *int-1* mutations were observed (Table 1). The pattern of insertion mutations around *int-2* in plaques did not seem to differ substantially

TABLE 1. Summary of *int-1* and *int-2* insertion mutations detected in samples from different stages of neoplastic development

Lesion type	No. of samples analyzed	No. (%) of mutations detected	
		<i>int-1</i> mutations	<i>int-2</i> mutations
Spontaneous tumors	27	7 (26)	12 (44)
Plaques	9	0 (0)	5 (56)
HOGs ^a	12	1 (8)	0 (0)
HOG-derived tumors	24	4 (17) ^b	2 (8)

^a HOG lines from 12 independent HANs were analyzed. In some cases more than one subline from a single HAN was examined.

^b Tumors from the 350 HOG line (with an *int-1* insertion mutation) were not included in these statistics.

FIG. 4. *int-2* insertion mutation during the development of a tumor in a premalignant HOG. (A and B) Southern blot comparison of 168B HOG DNA with DNA from a tumor that arose in a 168B HOG transplant (sample HT4 in Fig. 3). Sample DNAs were digested with *KpnI* and hybridized with ^{32}P -labeled *int-2* probe 39A (A) or MMTV(GR) LTR probe DM5 (B). Clonal exogenous MMTV restriction fragments that mark the major epithelial-cell population in the 168B HOG line (panel B, lane 2) are marked (open arrows). These fragments are also present in the 168B HOG-derived tumor (panel B, lane 3). A new clonal restriction fragment from an *int-2* mutation that occurred during tumor development in this lineage is also marked (solid arrow). This fragment is recognized by both *int-2* and MMTV probes (lanes 3). (C) Whole mount of a 168B HOG transplant illustrating the gross morphology of GR HOG transplants. Note that the sample has a normal (albeit hyperplastic) arborizing mammary tree and growth is confined to the mammary fat pad. The dark mass in the lower left is a lymph node.

from the pattern of insertion mutations in tumors (Fig. 3), although two of the insertion mutations (in samples P1 and P2) were observed upstream of the other integrations, probably in the region described as *int-P* (27, 33).

Insertion mutation of *int-1* in a premalignant HAN. The HANs studied in these experiments were all transplanted at least once prior to analysis. This procedure served two functions. First, the growth characteristics of the HANs upon transplantation ensured that the morphologically identified lesions were, in fact, premalignant hyperplasias rather than small incipient tumors. Second, the transplantation procedure increased the tissue mass to a level at which DNA analysis was possible. In addition, many transplanted HANs were halved or quartered and transplanted into more than one recipient fat pad. By this manipulation, a rare phenomenon observed in all sublines established from a single HAN could be presumed to result from its occurrence during noduligenesis. One noteworthy HAN, designated 350, had an insertion mutation of the *int-1* gene. HAN 350 was halved and transplanted into two different host fat pads to generate two independent HOG sublines, 350A and 350B. The morphologies of the 350A and 350B sublines were typical of most GR HOGs (Fig. 1 and 4C). However, when DNAs from the two sublines were analyzed for insertion mutations, both the 350A and 350B sublines were observed to have a common insertion mutation of the *int-1* locus (Fig. 5A). Other transplants and HOG-derived tumors from the 350 series were examined, and all had the same insertion mutation. The provirus was integrated 0.8 kb upstream of the *int-1* gene with the 5' LTR proximal to the gene (Fig. 5B).

DISCUSSION

A number of genomic loci have now been implicated in mammary tumorigenesis by virtue of their frequent mutation with exogenous MMTV provirus (27). Probes for two of these loci, *int-1* and *int-2*, were used to study MMTV insertion mutagenesis during multistep mammary neoplastic development. The GR mouse strain was chosen for this study because it has two types of MMTV-induced premalignant lesions that can be biochemically analyzed: HANs and plaques. These lesions represent two alternate morphogenic pathways of neoplastic development. Both HANs and plaques are classified as intermediate-stage group B neoplasms in the nomenclature of Foulds (15). Group B neoplasms are characterized by the potential, under the proper physiological conditions, to (i) grow without qualitative changes in morphology or growth autonomy, (ii) develop focal subpopulations with increased growth autonomy, (iii) persist as indolent nongrowing neoplasms, or (iv) regress entirely. By the multistep model of mouse mammary tumor development (25), genetic changes consistently found in both group B neoplasms and tumors occur early in neoplastic development, potentially initiating the process. In contrast, genetic changes found only in tumors hypothetically occur during progression of early lesions to malignant tumors.

To determine (i) whether *int-1* or *int-2* is mutated at a specific stage of neoplastic development and (ii) whether correlates exist between lesion type and mutation of *int-1* or *int-2*, we analyzed a total of 27 spontaneous tumors from nonpregnant, nonlactating breeders, 9 plaques, 12 transplanted HANs (HOGs), and 24 HOG-derived tumors from the GR strain. The results of this study support the following conclusions.

First, the details of provirus insertion at the *int-1* and *int-2*

FIG. 5. MMTV insertion mutation in a HAN. HAN 350 from a virgin GR female was divided in half and transplanted into two recipient fat pads. A novel *int-1* restriction fragment was observed in both primary transplants, 350A HOG and 350B HOG (as well as in later serial transplants). (A) Southern blot analysis of normal control spleen DNA (lane 1) and both 350 HAN primary transplants (lanes 2 and 3). Sample DNAs were digested with *ScaI* and probed with 32 P-labeled *int-1* probe C. A new clonal *int-1* restriction fragment was observed in both sublines (arrows). (B) Restriction map of the 350 HAN MMTV insertion mutation. Only sites delineating restriction fragments recognized by probe C are shown. Abbreviations: B, *BglII*; S, *ScaI*; X, *XbaI*.

loci in the GR strain were consistent with the general principles established in reports on tumors from the C3H and BR6 strains (28, 30). There was no apparent clustering of integration sites at either locus. No proviruses were found within the coding regions of either gene. With one exception, all upstream proviruses were oriented with the 5' LTR proximal to the *int-1* or *int-2* coding sequences. The exception was, unexpectedly, in tumor GRTu7, the tumor we used in our cloning experiments. Sequence analysis of the 3' host-virus junction showed that the GRTu7 provirus was integrated at nucleotide 1187 in the sequence of Moore et al. (23) in a promoter insertion orientation (data not shown). Two spontaneous tumors had mutations of both *int-1* and *int-2*. These mutations may represent concerted activation of both proto-oncogenes in a clonal cell population, as proposed by Peters et al. for many BR6 tumors (32). Three tumors were identified with downstream insertion mutations in which the MMTV 3' LTR was proximal to the *int* gene. Although this type of integration has occasionally been seen in other retroviral insertion mutation systems, it has not previously been reported in the MMTV system (27).

Second, insertion mutagenesis of *int-1* and *int-2* was not stage specific. Mutation of the *int-1* locus was observed during HAN development in one sample and during progression of mutation-free HOGs to adenocarcinomas in four samples from three independently derived HOG lines. In terms of the two-step hypothesis of mammary tumor development postulated by DeOme and Medina (7), we observed *int-1* mutations during both noduligenesis (step 1) and tumorigenesis (step 2). Mutation of the *int-2* locus was observed during plaque development in five samples and during progression of HOGs to adenocarcinomas in two samples from two independently derived HOG lines. Other reports suggest that *int-1* and *int-2* are also mutated occasionally during the development of hormone-dependent tumors and during the progression of these tumors to hormone-independent tumors

in the GR and BR6 strains (22, 31). The plaques described in this report are small, pregnancy-dependent lesions detected by surgical examination of midpregnant mice and probably represent an earlier stage of neoplastic development than the hormone-dependent GR tumors referred to above, which are induced with estrone and progesterone in castrated mice (22, 35).

Third, no simple correspondence was recognized between lesion morphology and *int-1* or *int-2* mutation in the groups of lesions examined. *int-2* mutations, exclusively, were observed in more than 50% of the plaques analyzed. However, there were no apparent differences between plaques with *int-2* mutations and those without. The negative correlation between *int-1* mutations and plaque development may be significant, but must be confirmed with a much larger pool of samples. Lesions from the HAN pathway had a slightly higher frequency of *int-1* mutations than *int-2* mutations. HOG-derived tumors as a class had fewer insertion mutations than spontaneous tumors. The lack of clear correlations between lesion type and insertion mutations may simply reflect the crude lesion classification criteria used in this study. It may be possible in the future to further classify these broad groups into subgroups based on histological and/or immunohistochemical analysis and hence to uncover more precise correlations.

Finally, the mutation of *int-1* observed in a HAN supports the hypothesis that insertion mutation can occur in the earliest stages of mammary neoplastic development. This *int-1* mutation, present in a premalignant lesion with, perhaps, 10^5 epithelial cells, is the earliest direct observation of insertion mutation in the mammary gland to date. An *int-1* mutation has also been observed in a BALB/cfC3H HOG; however, this mutation was postulated to have occurred during transplantation because it was present in only one of three sublines from a single progenitor HAN (29). Numerous other transplanted HANs from four strains have been ana-

lyzed, with no evidence of *int-1* or *int-2* mutations (25, 29). On the basis of (i) the absence of clustering of MMTV *int-1* integration sites in tumors (27) and (ii) the scarcity of *int-1* and *int-2* mutations in HOGs, it is unlikely that the *int-1* mutations observed in the two HOG sublines from HAN 350 are from independent integrations of MMTV at the same site during transplantation of the two sublines.

In light of the *int* gene mutation in HAN 350, we propose that other *int*-class genes are frequently involved in nodulogenesis. If such genes exist, they may be involved in establishing the two prominent phenotypes of these lesions, namely, (i) their ability to persist and grow in vivo with only basal levels of mammotropic hormones, and (ii) their immortal characteristics during serial transplantation. The relative phenotypic and morphological simplicity of HANs, relative to tumors, may allow these types of correlations to be unambiguously made.

In all classes of lesions examined, numerous samples without detectable insertion mutations of either *int-1* or *int-2* were observed. Several hypotheses could account for these results. First, MMTV may affect neoplastic development in MMTV-induced tumors by mechanisms in addition to insertion mutation. For example, one or more viral gene products may have an effect on oncogenesis under the appropriate conditions. Second, insertion mutations affecting *int* gene transcription may have sufficient long-range effects that they can occur outside the region we were able to observe by using the Southern blot protocol. However, from previous reports (27) plus our provirus mapping, no proviruses have been detected in the distal portions of the *int-1* and *int-2* regions analyzed in this report. RNA studies could validate our statistics. Unfortunately, for most samples, insufficient tissue was available for this type of analysis. Theoretically, a few additional insertion mutations could potentially be detected by RNA analysis; however, it should be noted that minor errors in our statistics would not affect our major conclusions regarding the lack of stage specificity of *int* gene mutation and insertion mutation in HANs. Third, *int-3* from Gallahan and Callahan (17), *int-4* from Nusse (27), or other undiscovered *int*-class genes may also play a role in mammary neoplastic development in the GR strain, particularly in the development of HANs. Evidence from other systems strongly suggests that this will account for at least a portion of the insertion mutation-negative samples in this survey. These possibilities are not mutually exclusive and may all be factors in explaining the results of this survey.

In conclusion, the data suggest that *int-1* and *int-2* are two of many proto-oncogenes that can participate in mammary neoplastic development in the mouse. Each gene probably has an incremental affect on neoplastic development which can be substituted by activation of other specific proto-oncogenes. Insertion mutagenesis of *int-1* or *int-2* appears to be more a matter of chance than of necessity; the observed mutation frequencies are a function of the mouse strain, virus strain, and lesion type analyzed. Mutations of *int-1* or *int-2* are not stage specific and can occur at different stages of mammary neoplastic development, including the development of premalignant lesions and the progression of premalignant lesions to malignancy. We expect that *int-1* and *int-2* mutations will also be detected at other stages of neoplastic development, for example, during progression of plaques to hormone-dependent tumors and during metastasis of mammary tumors to the lung.

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