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Host Genetic Background Effect on the Frequency of Mouse Mammary Tumor Virus-Induced Rearrangements of the *int-1* and *int-2* Loci in Mouse Mammary Tumors

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The frequency with which *int-1* and *int-2* are rearranged in mouse mammary tumors by mouse mammary tumor virus (MMTV)-induced insertional mutagenesis is a consequence of the host genetic background. In 75% of C3H mammary tumors, *int-1* is rearranged by MMTV insertion, whereas only 30% of BALB/cfC3H tumors contain a virus-induced rearrangement of *int-1*. This difference is significant ($P < 0.005$) and could not be accounted for by the potentially additive effect of the genetically transmitted Mtv-1-encoded virus in C3H mice. Similarly, MMTV-induced rearrangement of the *int-2* gene in mammary tumors of the R111 mouse strain (59%) occurred at a significantly ($P < 0.025$) higher frequency than in BALB/cfR111 (25%) mammary tumors. Moreover, in BALB/cfR111 mammary tumors, there is evidence that rearrangement of *int-1* and *int-2* does not occur independently ($P < 0.025$). These results suggest that the long history of inbreeding for high tumor incidence of C3H and R111 mouse strains has selected for the fixation of host mutations which either complement the action of the particular *int* gene or affect the sensitivity of specific subpopulations of mammary epithelium to infection by particular strains of MMTV.

The C3H, GR, BR6, and R111 mouse strains have been inbred over the past 40 to 50 years for a high incidence of mammary tumors (for a review, see reference 27). Mice of each strain congenitally transmit highly infectious mouse mammary tumor virus (MMTV) through the milk to their offspring. In addition, the C3H (11, 25) and GR (12, 26) mouse strains each contain a genetically transmitted or endogenous MMTV provirus genome (Mtv-1 and Mtv-2, respectively) that encodes an infectious virus that is also expressed in the milk. Parous C3H females develop pregnancy-independent mammary tumors at 7 to 10 months of age (21). Similarly, C3H mice in which the horizontally transmitted MMTV has been removed also develop pregnancy-independent mammary tumors as a consequence of infection by the Mtv-1-encoded virus but in the second year of life (25). The GR, BR6, and R111 females have a high incidence of pregnancy-dependent mammary tumors, or plaques, which after one or more parities progress to a pregnancy-independent tumor (9, 21, 26). Squartini et al. (22) demonstrated that differences in the manner in which the disease progressed in C3H and R111 females were a function of the particular strain of exogenous virus. In their study, BALB/c mice, which have a low or zero incidence of spontaneous mammary tumors (21), developed a high incidence of pregnancy-independent tumors when infected with horizontally transmitted MMTV(C3H) and pregnancy-dependent tumors which progressed to pregnancy independence when infected with MMTV(R111). MMTV induces mammary tumors by acting as an insertional mutagen within mammary epithelial cells (5, 14). The expression of three cellular genes (designated *int-1*, *int-2*, and *hst*/K-*fgf*) which are normally not expressed in the mammary gland are activated as a consequence of the integration of an MMTV provirus genome into flanking cellular DNA sequences (5, 8, 14, 16, 20, 28). Expression of the *int-1* gene normally occurs during embryonic development and is required for the development of the midbrain (10, 23). The *int-2* and *hst*/K-*fgf* genes are structurally related and are members of the fibroblast growth factor family of genes (2, 4, 29). Published reports indicate that the frequency with which *int-1* and *int-2* are rearranged in MMTV-induced mouse mammary tumors appears to vary significantly between different high-incidence inbred mouse strains (for a review, see reference 15). For instance, 80% of C3H mammary tumors contain a virus-induced rearrangement of *int-1*, whereas a much smaller fraction (10%) of the tumors contain a rearrangement of *int-2*. In contrast, the *int-1* and *int-2* genes are rearranged in only 40 and 5%, respectively, of C3Hf mammary tumors (7). Approximately 70% of BR6 mammary tumors contain a virus-induced rearrangements of *int-1* and/or *int-2*. The *hst*/K-*fgf* gene is closely linked to *int-2*, and its expression was activated by an integrated MMTV provirus in two BR6 tumors (16). In GR mammary tumors, the *int-1* and *int-2* genes were rearranged by MMTV in 25 and 44% of the cases, respectively. The variability in the frequency with which these genes are activated in the different mouse strains led us to consider whether this was a function of the particular host genetic background or the strain of MMTV. In this report, we show that the frequency of MMTV(C3H)- and MMTV(R111)-induced rearrangements of *int-1* and *int-2* in mouse mammary tumors is a function of the host genetic background.

To approach this question, we have examined the *int-1* and *int-2* genes in mammary tumors obtained from parous C3H/OuJ and BALB/cfC3H mice by Southern blot analysis. The position of the viral insertion sites around the *int-1* and

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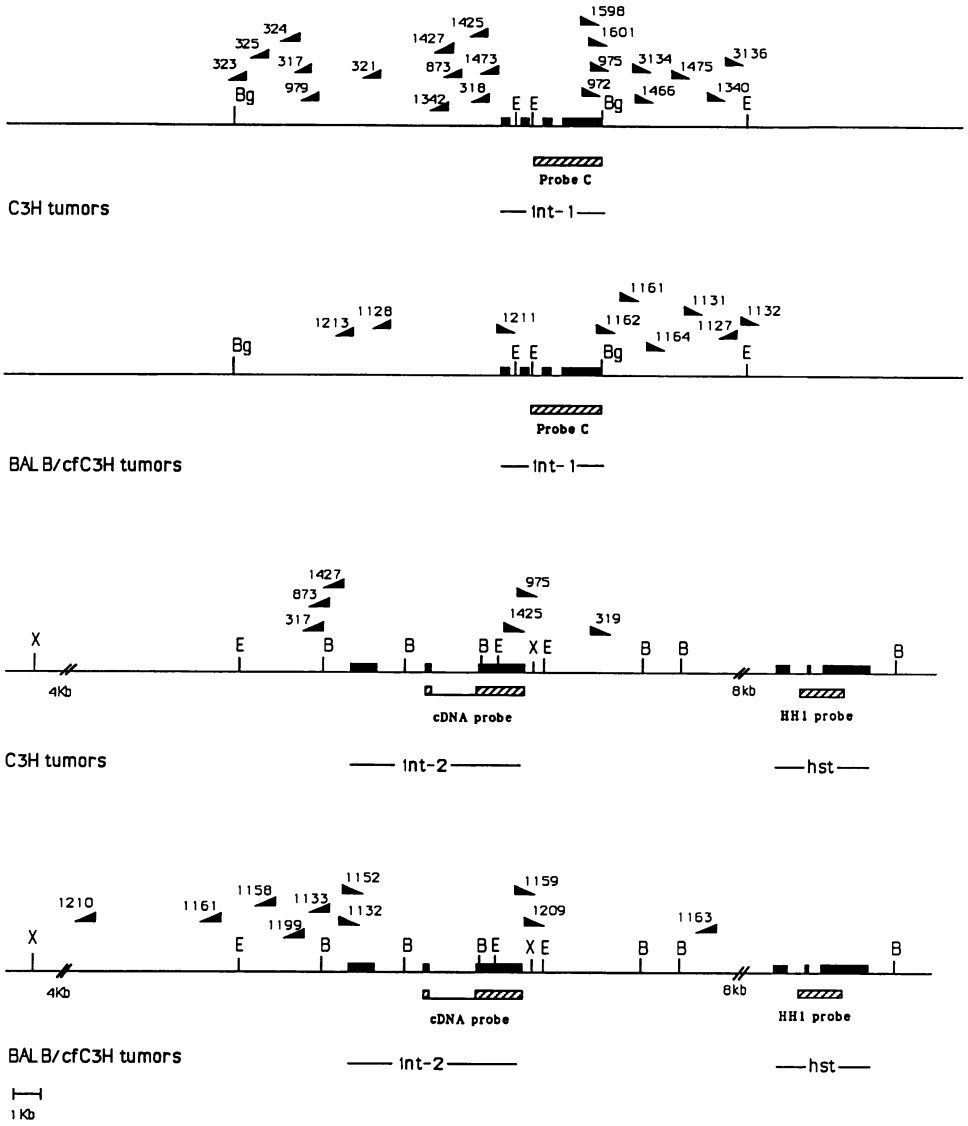

FIG. 1. Integration sites for MMTV at the *int-1*, *int-2*, and *hst*/K-*fgf* loci in C3H/OuJ and BALB/cfC3H mammary tumor DNAs. The arrowheads indicate the site and transcriptional orientation of the integrated MMTV provirus genome. The number above the arrowhead indicates the particular tumor. The tumor DNA restriction fragments which have been examined are defined by the restriction enzyme sites shown: E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; X, *Xba*I. The positions of the *int-1*, *int-2*, and *hst*/K-*fgf* exons are indicated by bars, and the regions corresponding to the probes (5, 14, 16) are shown below each restriction map.

int-2 genes, as well as the transcriptional orientation of the viral genome relative to the particular *int* gene, is shown in Fig. 1 for C3H/OuJ and BALB/cfC3H mammary tumors. In 21 of 28 (75%) of C3H/OuJ mammary tumors, an MMTV provirus genome was integrated in a region spanning 9 kb 5′ and 5 kb 3′ of the *int-1* gene (Table 1). However, in BALB/cfC3H mammary tumors, only 9 of 30 (30%) had a viral insertion in the same span of somatic DNA adjacent to *int-1*. On the basis of the exact test of Fisher, this represents a highly significant ($P = 0.004$) difference after a Bonferroni adjustment is applied.

We have considered several possible explanations for the difference in frequency of *int-1* rearrangements in mammary tumors of these mouse strains. One possibility is that in BALB/cfC3H tumors the majority of the viral integrations

TABLE 1. Frequency of MMTV integration at *int-1* and *int-2* in C3H/OuJ, BALB/cfC3H, R111, and BALB/cfR111 mammary tumors

Mouse strain (*n*)	Rearrangement of *int* genes (%)			
	int-1 only	*int-2* only	Both *int-1* and *int-2*	None
C3H/OuJ (28)	16 (57)	1 (3.5)	5 (18)	6 (21.5)
BALB/cfC3H (30)	7 (23)	7 (23)	2 (7)	14 (47)
R111 (29)	4 (14)	9 (31)	8 (27.5)	8 (27.5)
BALB/cfR111 (40)	6 (15)	3 (7.5)	7 (17.5)	24 (60)

occur at a greater distance from *int-1* than they do in C3H tumors. Although this seems unlikely on the basis of previous published results (15), it cannot be formally dismissed at this time. Another possibility is that the high frequency of virus insertions at *int-1* in C3H mammary tumors represents the sum of rearrangements induced by the horizontally transmitted MMTV(C3H) and the endogenous Mtv-1 viruses. Previous studies were not designed to distinguish the origin of the activating provirus (14, 15). The long terminal repeat (LTR) elements of MMTV(C3H) and Mtv-1, although highly related, differ significantly over a span of 100 bp in the U3 regions of these elements (1, 6). We have used synthetic oligomers corresponding to these two unique sequences to probe Southern blots of *Bgl*II-digested C3H normal liver and mammary tumor DNAs.

The Mtv-1 and MMTV(C3H) oligomers were 41 and 48 bases long, respectively. The sequence of each was Mtv-1, 5′-ATTAAGGCTTTGCCTTAGCTTTCTAAAGTTTGCT TGCGGTT-3′, and MMTV(C3H), 5′-TCTGCAAAAACTT ATGGCATGAGTTATTATGAATAGCCTTTATTGG CC-3′. The LTR and *int-1* restriction fragments were electrophoretically separated from plasmid DNA on agarose gels, purified, and labeled with [^{32}P]dCTP by the random primer technique (Boehringer-Mannheim kit). The oligomers were synthesized on an 8700 DNA synthesizer (Millipore) using cyanoethyl phosphoramidite chemistry and end labeled with [γ-^{32}P]ATP as described elsewhere (19). The digested cellular DNAs were separated electrophoretically on agarose gels and then transferred to a Genatran nylon membrane (Plasco) by using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the transfer medium. Prehybridization conditions for blots to be hybridized with random primed probes was carried out at 37°C for at least 2 h in 40% formamide–5× Denhardt's solution (3)–15% sodium dodecyl sulfate (SDS)–3× SSPE (20× SSPE is 3M NaCl, 0.2 M NaH_2PO_4 [pH 7.4], 0.02 M EDTA)–2.5% dextran sulfate–0.001 M sodium phosphate (pH 6.5). Unreacted probe was removed from the membranes by washing two times, 30 min each, with 0.3× SSC–0.5% SDS at 65°C. Prehybridization and hybridization with oligomeric probes was performed at 35°C for 24 h in 2× SSC–2× Denhardt's solution–0.5% SDS. The hybridization solution contained 3 × 10^6 cpm of ^{32}P-end-labeled probes. Filters were washed two times for 10 min at room temperature and one time at 42°C with 2× SSC–0.5% SDS. The filters were exposed to Kodak X-Omat AR film at −70°C with a Cronex intensifier screen.

The MMTV(C3H) probe specifically recognizes restriction fragments corresponding to acquired proviruses detected by the MMTV LTR probe in C3H tumor DNAs (Fig. 2, lane 3). The Mtv-1 probe detects only restriction fragments corresponding to the endogenous Mtv-1 and Mtv-6 proviruses (Fig. 2, lane 4, and data not shown). Since none of the restriction fragments corresponding to acquired MMTV provirus genomes reacted with this probe, we conclude that Mtv-1 virus does not contribute to the increased frequency of virus-induced *int-1* gene rearrangements in C3H mammary tumors. A more likely explanation is that the rigorous inbreeding of C3H mice for high tumor incidence and short tumor latency has led to the fixation of a mutation(s) in the host genetic background which either complements the action of *int-1* during mammary tumor development or broadens the target population of cells susceptible to MMTV(C3H) infection during mammary gland development.

The frequencies of virus insertions around the *int-2* gene in

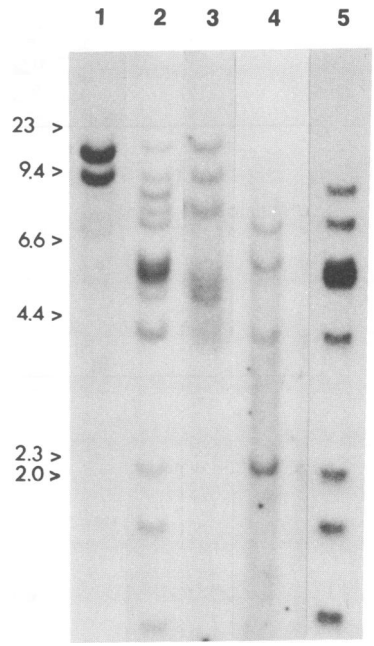

FIG. 2. Southern blot analysis of the *int-1* locus in a C3H/OuJ mammary tumor DNA. Southern blot of *Bgl*II-digested cellular DNA from C3H/OuJ mammary tumor DNA 1427 (lanes 1 through 4) and normal liver DNA (lane 5) was hybridized with the following recombinant DNA probes: lane 1, *int-1* (see Fig. 1); lanes 2 and 5, MMTV LTR; lane 3, exogenous MMTV(C3H) LTR oligomer; and lane 4, endogenous Mtv-1 oligomer.

C3H/OuJ and BALB/cfC3H tumors, in contrast to insertions around *int-1*, were not significantly different (6 of 28, 21.5%, and 9 of 30, 30%, respectively) (Table 1). In addition, we examined the *hst*/K-*fgf* gene for evidence of virus-induced rearrangement and expression. One BALB/cfC3H tumor DNA (1163) contained an integrated provirus genome near the *hst*/K-*fgf* gene but in the opposite transcriptional orientation (Fig. 1 and 3A). Northern (RNA) blot analysis of poly(A)$^+$ RNA from this tumor demonstrated the presence of a 3.2-kb species of *hst*/K-*fgf* RNA but no evidence of *int-2* RNA (Fig. 3B). One C3H tumor DNA (319) contained a provirus genome 2.5 kb 3′ of *int-2* and in the same transcriptional orientation (Fig. 1). This tumor expressed both *int-2* and *hst*/K-*fgf* RNA species (Fig. 3B). Since no additional provirus genomes were detected 3′ of the *hst*/K-*fgf* gene the mechanism by which both cellular genes are activated by a single provirus genome is unclear at the present time. One possibility is suggested by the presence of two minor species (3.5 and 4.0 kb) of *hst*/K-*fgf* RNA which were detected and are larger than the predominant 3.2-kb RNA species. These two RNA species may represent *hst*/K-*fgf* transcripts initiated in the MMTV LTR. Although their sizes are smaller than would be expected based on the location of the provirus genome, there may be cryptic splice signal sequences within the region between the viral genome and *hst*/K-*fgf*. Alternatively, it has been suggested (16) that the region between *int-2* and *hst*/K-*fgf* contains *cis*-acting suppressor sequences which when disrupted by an MMTV provirus allow *hst*/K-*fgf* expression from its normal promoter. Irrespective of the mechanism by which expression of *hst*/K-*fgf* is activated by MMTV, these results support the conclusions (14) that activation of this gene can contribute to mammary tumori-

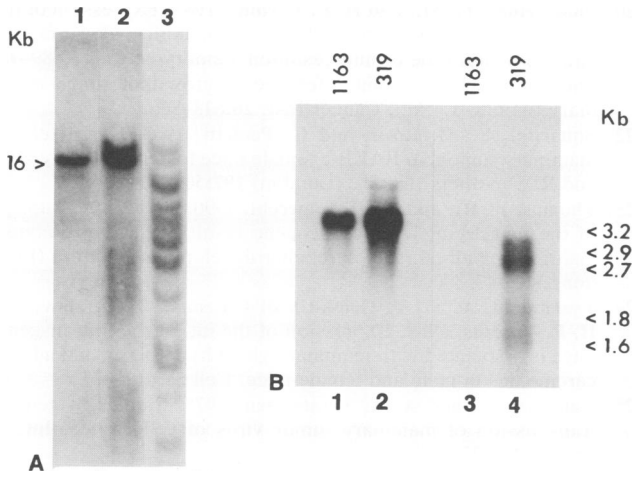

FIG. 3. (A) Southern blot analysis of the *hst*/K-*fgf* locus in a BALB/cfC3H tumor. *Bam*HI-digested genomic DNA from a BALB/cfC3H normal liver (lane 1) and mammary tumor (1163) (lanes 2 and 3) was hybridized with the following probes: HH1 (lanes 1 and 2) and MMTV-*gag* (lane 3). (B) Northern blot analysis of poly(A)$^+$ RNAs from C3H mammary tumor 319 and BALB/cfC3H mammary tumor 1163. RNA (5 μg) was separated by electrophoresis on a 1% agarose-formaldehyde gel. A blot of the gel was hybridized with HH1 (lanes 1 and 2) or *int-2* cDNA probes (lanes 3 and 4) labeled with ^{32}P by the random primer kit.

genesis. Although *int-2* and *hst*/K-*fgf* are structurally related, further studies will be required to determine the biological basis for the more frequent activation of the *int-2* gene by MMTV during mammary tumorigenesis.

To determine whether the effect of the host genetic background on the frequency of *int* gene rearrangement in mammary tumors is unique to C3H mice, we have extended the analysis to include R111 and BALB/cfR111 mammary tumors. By using the same restriction enzymes and probes, 41.5% (12 of 29) and 58.5% (17 of 29) of the R111 tumors had a virus insertion near *int-1* and *int-2*, respectively (Table 1). Thus, while insertional rearrangement of the *int-1* locus appears to be favored over *int-2* in the C3H mouse genetic background ($P < 0.002$ on the basis of McNemar's test with the Bonferroni adjustment), there is no evidence of a significant difference in the frequency with which the two *int* loci are rearranged in RIII mammary tumors. However, the high frequency of virus insertions at *int-2* (58.5%) in R111 tumors was significantly ($P < 0.025$ after Bonferroni adjustment for the four tests) reduced to 25% (10 of 40) in BALB/cfR111 tumors, whereas the frequency of *int-1* rearrangements was not significantly different in tumors from these two mouse strains (42 and 33%, respectively) (Table 1). There was no evidence of MMTV-induced rearrangement of the *hst*/K-*fgf* gene in either R111 or BALB/cfR111 tumors. Therefore, it again seems likely that the inbreeding regimen used to develop and maintain the R111 mouse strain has led to the fixation of a host mutation(s) which complements the action of an activated *int-2* gene during tumor development.

The high frequency with which both *int-1* and *int-2* are activated by MMTV(R111) integration in BR6 mammary tumors is consistent with an association between the activation of both genes during the progression from normal mammary epithelium through pregnancy-dependent tumors to pregnancy-independent tumors (18). Moreover, activation of *int-2* appears to occur frequently in the pregnancy-dependent phase of BR6 mammary tumor development, prior to the progression to pregnancy independence (17). These observations led us to consider whether rearrangement of *int-1* and *int-2* occur independently in the R111 and BALB/cfR111 tumors. The different sets of tumors were analyzed separately. There was no evidence of dependence between the rearrangement of *int-1* and *int-2* in R111, C3H/OuJ, or BALB/cfC3H mammary tumors, although larger sample sizes may reveal statistically significant associations. However, there is a positive association between *int-1* and *int-2* rearrangements in BALB/cfR111 tumors ($n = 40$) (Table 1). The phi coefficient is 0.462, with a P value from Fisher's exact test of 0.006, or 0.024 when the Bonferroni correction for the four independent hypotheses is applied. Here, a positive phi indicates that if one *int* gene is rearranged, the other is more likely to be rearranged as well. In considering the implications of this association, it may be pertinent that FVB/N mice containing a MMTV LTR-*int-2* transgene develop pregnancy-dependent mammary hyperplasia (13), whereas (C57BL/6 × SJL)F_1 mice containing the MMTV LTR-*int-1* transgene develop pregnancy-independent mammary hyperplasias (24). These observations suggest that in some BALB/cfR111 tumors there may first be a selection for activation of the *int-2* gene, followed by activation of the *int-1* gene during the progression toward pregnancy-independent mammary tumors. However, it is also clear from the similar frequency with which *int-1* and *int-2* are rearranged in BALB/cfC3H and BALB/cfR111 tumors that activation of either gene cannot be exclusively linked to either pregnancy-dependent or pregnancy-independent mammary tumors. This raises the possibility that the two different virus strains selectively infect different subpopulations of mammary cells in BALB/c mice and that activation of *int-1* or *int-2* has different consequences in these subpopulations.

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