Expression of the *int-1* and *int-2* Loci in Endogenous Mouse Mammary Tumor Virus-Induced Mammary Tumorigenesis in the C3Hf Mouse

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The *int-1* locus appears to be involved in over 80% of C3H exogenous mouse mammary tumor virus (MMTV)-induced mouse mammary tumors, and the *int-2* locus appears to be involved in approximately 10% of these tumors. Analysis of 46 C3Hf mammary tumors resulting from endogenous, rather than exogenous, MMTV infection revealed that only 41% expressed *int-1* RNA, while 2% expressed *int-2* RNA. Our results suggest that in addition to the *int-1* and *int-2* loci, other loci may be involved in endogenous-MMTV-induced mammary tumors of the C3Hf mouse.

Inbred mice of the C3H Heston strain express high titers of mouse mammary tumor virus (MMTV) in their milk, and approximately 90% of C3H female offspring develop mammary tumors between the ages of 7 and 10 months as a result of horizontal infection by exogenous MMTV (2, 17, 18). MMTV is also transmitted vertically in the form of integrated copies of MMTV proviral DNA, which have been found in the germ line of every inbred mouse strain tested (4). Depending on the strain of mouse, these endogenous MMTV proviral DNA copies may or may not be activated to produce endogenous MMTV. Foster nursing of C3H offspring on virus-free mothers of other strains results in an incidence of mammary tumor development of 20 to 40% at 18 to 24 months of age (1, 2). These late-occurring mammary tumors, which develop only after multiple pregnancies and independently of exogenous MMTV infection, result from activation of the MMTV provirus which is present at the *Mtv-1* locus in the germ line of these C3H foster-nursed (C3Hf) mice (32). The activation of this provirus results in endogenous MMTV production with consequent endogenous virus infection of the mammary gland (5, 9).

All C3Hf mammary tumors examined contain integrated MMTV genomes in addition to the endogenous copies present in the germ line, indicating that tumorigenesis in the C3Hf mouse is always associated with the reintegration of the MMTV provirus generated from the *Mtv-1* locus, just as tumorigenesis in the C3H mouse is always associated with the integration of exogenous MMTV (21). In over 80% of C3H exogenous-virus-induced mammary tumors at least one of the additional MMTV genomes is integrated near the cellular gene locus designated *int-1* (10, 21), and in approximately 10% of the tumors integration is near the locus designated *int-2* (10, 23). The activation of RNA transcription of the *int-1* and/or *int-2* loci by MMTV proviral insertion is believed to be an early step in the transformation process (24). Since mammary tumors which develop in the C3Hf mouse as a result of endogenous virus infection differ from the exogenous-virus-induced tumors of the C3H mouse with regard to time of development, incidence, type of infecting virus, and dependence on multiple pregnancies, we examined C3Hf mouse mammary tumors to determine if activation of RNA transcription of the *int-1* and *int-2* loci by

MMTV proviral integration is involved in the transformation process in these mice.

High-molecular-weight DNA (9) and total cellular RNA (3) were prepared from 46 mammary tumors which arose spontaneously over a period of 3 years in a C3Hf inbred mouse colony. This colony was initially established in 1984 by Jane Holben at the Institute for Medical Research, Camden, N.J. After being allowed to breed until 1 year of age, first-litter female C3Hf mice were then maintained as retired breeders and monitored for the development of mammary tumors. Approximately 25% of the C3Hf female mice developed mammary tumors between 16 and 18 months of age. All C3Hf mammary tumors were carefully screened as previously described (9) to be certain that only endogenous MMTV was present.

Expression of the *int-1* gene was determined by using a 2.5-kilobase (kb) *EcoRI*-*Bam*HI fragment called probe *int-1-c* (20, 33), which was derived from the third and fourth exons of *int-1* (25). *int-2* RNA expression was examined by using a 1.8-kb *Bam*HI-*Hind*III fragment representing most of the 3' exon of *int-2* (designated probe *int-2-f*) (6, 25) and a 2.0-kb *Sac*I fragment representing the 5' exon of *int-2* (called probe *int-2-c*) (6, 25). Of the 46 C3Hf mammary tumors examined for *int*-specific RNA transcription, 18 tumors expressed only *int-1*-specific RNA, 1 tumor expressed only *int-2*-specific RNA, and 1 tumor expressed both *int-1*- and *int-2*-specific RNAs. Figure 1 shows the results of an experiment in which 20- μ g portions of C3Hf mammary tumor and liver total RNAs, which were isolated by the guanidine-isothiocyanate procedure (3), were fractionated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde and were transferred to nitrocellulose by the Northern blot (RNA blot) procedure (31). Hybridizations were performed in the presence of 3×10^6 cpm of [32 P]dCTP-labeled nick-translated probe (27) as previously described (9).

C3Hf mammary tumors produced the MMTV-specific RNA transcripts of 9.0, 3.8, and 1.7 kb (Fig. 1A, lanes b through g) (28, 34). The 9.0-kb transcript serves as the genome for progeny virions and encodes the *gag* and *gag-pol* polypeptide precursors. The 3.8-kb MMTV species is a spliced transcript that encodes the virion envelope glycoproteins. The 1.7-kb MMTV RNA presumably is the spliced transcript that contains sequences derived from the long

FIG. 1. Expression of MMTV, *int-1*, and *int-2* transcripts in C3Hf mouse mammary tumors and liver. RNAs from C3Hf mammary tumors 16 (lane b), 17 (lane c), 18 (lane d), 15 (lane e), 13 (lane f), and 12 (lane g) and from C3Hf liver (lane a) were analyzed by Northern blotting and hybridization to a 32 P-labeled (27) MMTV probe representative of the entire viral genome (A), to the *int-1*-c probe (B), and to the *int-2*-f probe (C). Numbers indicate the sizes of the RNA transcripts seen. The sizes of the indicated RNAs were determined by running on the same gels a 0.24- to 9.5-kb RNA ladder (purchased from Bethesda Research Laboratories) which was detected by ethidium bromide staining under UV illumination.

terminal repeat region of the MMTV provirus (34) which encode the MMTV open reading frame protein (7, 26). All C3Hf mouse livers such as the one shown in Fig. 1A, lane a, were negative for MMTV RNA transcription. Figure 1B shows the same samples as in panel A hybridized to the *int-1*-c probe. Lanes b, c, d, e, and g represent RNA from 5 of the 19 C3Hf mammary tumors which expressed the *int-1*-specific 2.6-kb RNA transcript (19–21). The mammary tumor represented in lane f and the liver in lane a were negative for *int-1* RNA transcription. In Fig. 1C there are both 2.9- and 1.8-kb *int-2*-specific RNA transcripts (16, 19) expressed by the C3Hf mammary tumor represented in lane b. This same mammary tumor also expressed the *int-1*-specific 2.6-kb RNA transcript (Fig. 1B, lane b). A second mammary tumor (data not shown) also expressed the *int-2*-specific RNA transcript of 2.9 kb.

Hybridization of the *int-1*-c probe to the *Bgl*III and *Eco*RI restriction enzyme digests of C3Hf mammary tumor DNA (Fig. 2A and B, respectively), which represent a genomic distance of over 15 kb covering an area where nearly 100% of the exogenous MMTV proviral insertions at the *int-1* locus in C3H mice have been reported (19), revealed the presence of *int-1* integrations in 14 of 46 C3Hf mammary tumors. Figure 2A, lanes b through m, represents 12 of 46 C3Hf mammary tumors examined which were found to contain *int-1* integrations after *Bgl*III digestion and hybridization to the *int-1*-c probe. Figure 2B, lanes b through e, represents four C3Hf mammary tumors which were found to contain *int-1* integrations after *Eco*RI digestion and hybridization to the *int-1*-c probe. Two of these tumors (lanes c and d) also revealed *int-1* integrations after *Bgl*III digestion (Fig. 2A, lanes i and

FIG. 2. Rearrangement of *int-1* and *int-2* loci in C3Hf mammary tumor DNA. Mammary tumor and liver cellular DNAs (20- μ g portions) were digested with *Bgl*III (A and C) or *Eco*RI (B), run on 0.8% agarose gels (15), Southern transferred (30), and hybridized (9) to a 32 P-labeled (27) *int-1*-c probe (A and B) or to the *int-2*-c probe (C). The cellular DNAs in panel A were from mammary tumors 16 (lane b), 17 (lane c), 18 (lane d), 45 (lane e), 37 (lane f), 24 (lane g), 3 (lane h), 36 (lane i), 8 (lane j), 47 (lane k), 32 (lane l), and 28 (lane m); those in panel B were from mammary tumors 35 (lane b), 47 (lane c), 36 (lane d), and 19 (lane e); and those in panel C were from mammary tumors 16 (lane a) and 2 (lane b). C2Hf mouse liver DNA is represented in panel A, lanes a and n; panel B, lanes a and f; and panel C, lane c. Sizes indicated on the left were determined from the positions of fragments of *Hind*III-digested lambda DNA which were used as markers. Arrows indicate the positions of the *int-1*- and *int-2*-specific rearranged fragments.

k). The normal-sized allele of *int-1* after *Bgl*III digestion and hybridization to the *int-1*-c probe is 14 kb, while after *Eco*RI digestion it is 8 kb. The alleles with new sizes resulting from MMTV proviral integration are represented by arrowheads in Fig. 2.

To detect MMTV proviral integration at the *int-2* locus, C3Hf mammary tumor and liver DNAs from 46 C3Hf mice were analyzed with the nick-translated *int-2*-f probe as well as an *int-2*-c probe. *Eco*RI digests were also examined with an *int-2*-j probe derived from the region downstream of the *int-2* transcriptional units (25). Using each of these three different probes, which are directed toward three different regions of the *int-2* locus (25), for hybridization against *Eco*RI, *Bgl*III, and *Hind*III digests which together encompass over 20 kb of the *int-2* domain (25), only two C3Hf mammary tumors which had integrated MMTV proviral DNA at this locus were detected. Figure 2C, lanes a and b, represents the two mammary tumors which showed *int-2* integrations after *Bgl*III digestion and hybridization to the *int-2*-c probe. *int-2* integrations in these two tumors were also seen in *Hind*III and *Eco*RI digests which were hybridized to either the *int-2*-c or *int-2*-f probe.

Like mammary tumors induced by C3H exogenous MMTV infection (21), mammary tumors resulting from endogenous MMTV infection appear to be of clonal origin (5, 9). The variable intensities of the bands representing new integrations at the *int* loci in C3Hf mammary tumors (Fig. 2) and the lesser intensity of these bands as compared with the bands representing the normal *int-1* or *int-2* allele have also been seen in mammary tumors caused by exogenous MMTV (21). These variations in band intensities have been ascribed to heterogeneity in the cell population in the excised tumor sample, since normal cells from stromal tissue or adjacent mammary gland are difficult to exclude from the sample, rather than to a lack of clonality (21).

The presence of integrated MMTV DNA sequences in the

TABLE 1. Involvement of the *int-1* and *int-2* loci in C3Hf mouse mammary tumors

Tumor	Presence of MMTV provirus ^a /expression of RNA ^b	
	<i>int-1</i>	<i>int-2</i>
2	-/-	+/+
3	+/+	-/-
4	-/-	-/-
5	-/-	-/-
6	-/-	-/-
7	-/-	-/-
8	+/+	-/-
9	-/+	-/-
10	-/-	-/-
11	-/-	-/-
12	-/+	-/-
13	-/-	-/-
14	-/-	-/-
15	-/+	-/-
16	+/+	+/+
17	+/+	-/-
18	+/+	-/-
19	+/+	-/-
20	-/-	-/-
21	-/-	-/-
22	-/+	-/-
23	-/-	-/-
24	+/+	-/-
25	-/-	-/-
26	-/-	-/-
27	-/-	-/-
28	+/+	-/-
29	-/+	-/-
30	-/-	-/-
31	-/-	-/-
32	+/+	-/-
33	-/-	-/-
34	-/-	-/-
35	+/+	-/-
36	+/+	-/-
37	+/+	-/-
38	-/-	-/-
39	-/-	-/-
40	-/-	-/-
41	-/-	-/-
42	-/-	-/-
43	-/-	-/-
44	-/-	-/-
45	+/+	-/-
46	-/-	-/-
47	+/+	-/-

^a From Southern blots of restriction enzyme-digested DNA from 46 C3Hf mammary tumors hybridized with probes specific for the *int-1* and *int-2* domains.

^b From Northern blots of RNA from 46 C3Hf mammary tumors hybridized with the *int-1-c* and *int-2-f* probes.

int loci was confirmed by rehybridizing the restriction enzyme digest blots after removal of the *int* (21) probe with an MMTV probe representing the entire MMTV genome. For each sample an additional band in the autoradiogram that was visualized with the *int* probes comigrated with a band visualized by the MMTV probe.

In addition to the *int-1* and *int-2* loci, an *int* locus designated *int-3* has been implicated in mouse mammary tumorigenesis, specifically in the feral Czech II mouse strain (10, 11). All 46 of the C3Hf mammary tumors described here have been found to be negative for *int-3* integrations, and no *int-3*-specific RNA transcription has been detected in any of these 46 C3Hf mammary tumors.

Table 1 summarizes the involvement of the *int-1* and *int-2* loci in C3Hf mouse mammary tumorigenesis. Of the 14 C3Hf mammary tumors in which *int-1* integrations were detected, all expressed *int-1*-specific RNA transcripts. Both of the two C3Hf mammary tumors in which integration at the *int-2* site was seen expressed *int-2*-specific RNA transcripts. One mammary tumor expressed both *int-1*- and *int-2*-specific RNA and contained both *int-1* and *int-2* integrations, as others have reported for mammary tumors resulting from MMTV infection (13, 25). RNA expression at specific *int* sites without corresponding proviral integrations at these sites, as reported here for five C3Hf mammary tumors and the *int-1* locus, has also been reported by others (13, 25). The inability to detect proviral integrations at *int* loci at which *int*-specific RNA transcription is detected may be a result of the large distances between integration sites and activated sequences, as has recently been shown for the *int-2* domain in the GR mouse (22, 29). The results of this study strongly suggest that in addition to *int-1* and *int-2* involvement in C3Hf mouse mammary tumorigenesis, other sites which appear not to include *int-3* may be involved, since over half of the C3Hf mammary tumors examined did not express *int-1*-, *int-2*-, or *int-3*-specific RNA. These results reinforce the findings of others which suggest that numerous *int* loci may play a role in the tumorigenic process in the MMTV system (8, 12–14, 19).

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