Eur J Cancer, Vol. 26, No. 1, pp. 9–15, 1990.
Printed in Great Britain

0277–5379/90$3.00 + 0.00

DNA Rearrangements of the *int* Region in Spontaneous Mouse Mammary Tumors of SHN/S and SLN/S Mice

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Abstract—*SHN and SLN mice originating from the same Swiss albino stock are genetically very close to each other. The incidence and latent period of mammary tumor development in SHN mice were higher and shorter than those in SLN. To elucidate these differences in the behavior of mammary tumorigenesis, the frequency of insertion of mammary tumor viral genes within the* int-1 *and* int-2 *regions in spontaneous mammary tumors from their two substrains, SHN/S and SLN/S, were compared. The frequency of provirus integration into either* int-1 *or* int-2 *in DNAs from mammary tumors was* 52% (11/21) *in SHN/S and* 45% (5/11) *in SLN/S. The frequency of insertion within* int-1 *or* int-2 *could not account for the different susceptibilities of SHN/S and SLN/S.*

INTRODUCTION

SHN and SLN are strains of mice with a high and relatively low spontaneous mammary tumor incidence respectively, derived from the same stock of Swiss albino mice [1, 2]. The genetic marker patterns of the SHN and SLN strains have been extensively studied and they are similar except for hemoglobin beta chain (Hbb) and glucose phosphate isomerase-1 (Gpi-1) markers [3]. These two strains of mice have been comparatively examined for several characteristics: reproductivity [1], the time of onset of hyperplastic alveolar nodule (HAN) formation [4], the level of hormones in serum [5], the responsiveness of mammary gland cells to lectin [6], the incidence of diseases such as uterine adenomyosis [7, 8] and the expression of mouse mammary tumor virus (MMTV) antigens in various organs [9].

The incidence of mammary tumor in SLN, however, has recently become close to that in SHN, but the latent period of the tumors is still significantly longer than in SHN [7].

Both strains carry mouse mammary tumor virus (MMTV), which is transmitted from mother to offspring, causing spontaneous mammary tumors [1, 2, 9]. Moreover, in the SHN strain, the Mtv-4 locus controls early mammary tumor development [10]. Mammary tumorigenesis by MMTV, which does not carry an oncogene, takes place due to the insertion of viral DNA into specific cell loci designated *int*-1 and *int*-2 and the activation of adjacent cellular genes [11–17]. It is of interest to know whether differences in the insertional mutagenesis of the *int*-1 and/or *int*-2 loci between SHN and SLN strains would contribute to differences in mammary tumorigenesis. In this study, tumors from both strains acquired additional MMTV genomes in varying copy numbers. The newly acquired viral genomes were integrated with a high frequency into the *int*-1 and/or the *int*-2 regions. There was, however, no significant difference in the frequency of insertion betweeen SHN and SLN strains.

MATERIALS AND METHODS

1. *Mice and mammary tumors*

The SHN and SLN strains of mice were originally obtained at the 34th and 29th generations, respectively. They have been maintained for over 30 generations by brother–sister mating in our laboratory. They were designated as SHN/S and SLN/S, respectively. Mammary tumors from SLN/Mei mice

Accepted 11 August 1989.
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were kindly provided by Dr Nagasawa (Meiji University, Tokyo). The mammary tumors and livers from SHN/S and SLN/S mice were excised and stored at −80°C until extraction of DNA.

2. *DNA extraction*

High molecular weight DNA was extracted from livers and mammary tumors according to Maniatis *et al.* [18].

3. *Restriction enzyme digestion and agarose gel electrophoresis*

DNA (5 μg) was digested with various restriction endonucleases, electrophoresed in 0.7% agarose gels and transferred to nitrocellulose filters, for Southern blots [19].

4. *Nick translation and Southern blot analysis*

Hybridization probes were MMTV, LTR, *env*, *gag-pol*, *int*-1c, *int*-2c, *int*-2c and *int*-2j. MMTV *gag-pol*, *int*-2c and *int*-2e were kindly provided by Dr C. Dickson (Imperial Cancer Research Fund Laboratories, London, U.K.) [14]. MMTV LTR and *int*-1c were kindly provided by Dr R. Nusse (Netherlands Cancer Institute, Amsterdam, Netherlands) [12]. MMTV *env* and *int*-2j were a gift from Dr A. Murakami (Institute for Virus Research, Kyoto University, Kyoto, Japan). All probes were labeled with ^{32}P-labeled dCTP by nick translation [20]. The filters were hybridized with the ^{32}P-labeled probes at 65°C for 18 h. Filters were washed sequentially in 2 × SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) for 5 min at room temperature, two times in 0.1 × SSC plus 0.1% SDS for 20 min at 65°C and in 2 × SSC for 30 min at room temperature.

RESULTS

1. *Incidence of mammary tumors in SHN/S and SLN/S*

The incidence of mouse mammary tumor in SHN/S mice was 93% (42/45) in breeders and 81% (114/140) in virgins, while that in SLN/S was 78% (14/18) in breeders and 65% (43/66) in virgins (Fig. 1). The modal age of mammary tumor developments in breeders and virgins of SHN/S mice was about 6 and 7 months, respectively, whereas that in breeders and virgins of SLN/S mice was about 11 and 13 months, respectively. Individual SHN/S mice frequently developed two to four mammary tumors, but SLN/S mice developed only one or rarely two tumors (Table 1).

2. *Endogenous MMTV proviruses in normal liver DNA*

DNA was extracted from livers of SHN/S and SLN/S mice, treated with *Eco*RI and hybridized with the MMTV *env*, *gag-pol* and LTR probes.

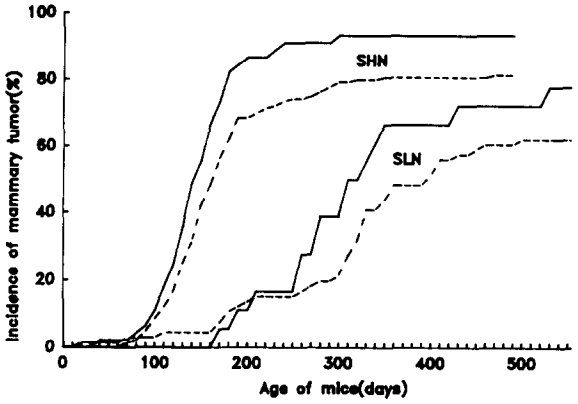

Fig. 1. *Incidence of mammary tumors in SHN/S and SLN/S:* — *breeder,* --- *virgin.*

*Eco*RI cleaves MMTV proviral DNA at a single site and yields two unique cell-virus junction fragments for each complete proviral genome [21]. As shown in Fig. 2A, *Eco*RI digestion of liver DNA in SHN/S yielded five fragments which hybridized with the MMTV *env* prove and four fragments which detected with the MMTV *gag-pol* probe. Using the MMTV LTR probe, a new 1.7 kb fragment was detected, indicating that SHN/S mice contain five endogenous MMTV proviruses, probably four with the complete genome and one incomplete genome lacking the *gag-pol* region. The SLN/S mice, however, contained two additional fragments, of 6.5 kb and 5.1 kb (Fig. 2B). Hence, SLN/S mice contain five complete endogenous MMTV proviruses plus one partial proviral unit lacking the *gag-pol* region.

3. *Additional integration of MMTV proviruses into mouse mammary tumor DNAs and the rearrangement of the* int *regions*

DNA was extracted from 21 and 11 mammary tumors from 10 SHN/S and 10 SLN/S mice, respectively (Table 1). The tumor DNA from both strains contained a varying number of additional *Eco*RI fragments which were different in individual tumors (data not shown). Proviral integrations within approximately 20 kb region, including the *int*-1 transcriptional unit, were detected by endonuclease, *Eco*RI or *Bgl*II and the *int*-1c probe. All MMTV integrations at *int*-1 were found in this 20 kb region as described previously [11, 16]. Nine of 21 (43%) SHN/S mammary tumors (Table 1, Fig. 3a and b) and four of 11 (36%) SLN/S mammary tumors were found to retain the rearrangements in the *int*-1 region (Table 1, Fig. 4a and b). Proviral integrations within an approximately 22 kb region, including the *int*-2 transcriptional unit were detected. Most MMTV integrations at *int*-2 have been found in this region [14, 16]. Three of 21 (14%) SHN/S mammary tumors (Table 1, Fig. 3c) and two of 11 (18%) SLN/S mammary tumors (Table 1, Fig. 4c

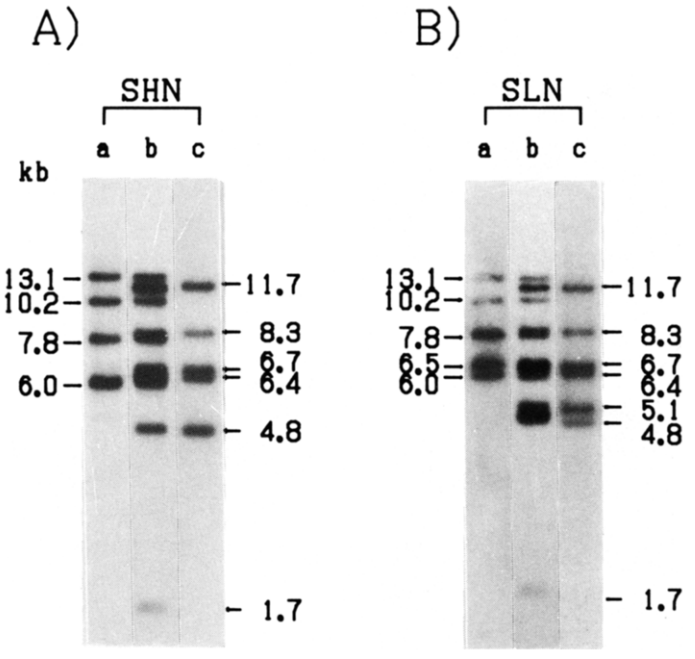

Fig. 2. Southern blot hybridization analysis of endogenous MMTV provirus in SHN/S (A) and SLN/S (B). Eco*RI-digested liver DNA was hybridized with the following probes: the MMTV* gag-pol *(a),* LTR *(b) and env (c). Fragment size, indicated in kilobases, was estimated by Hind III digests of lambda DNA as standards.*

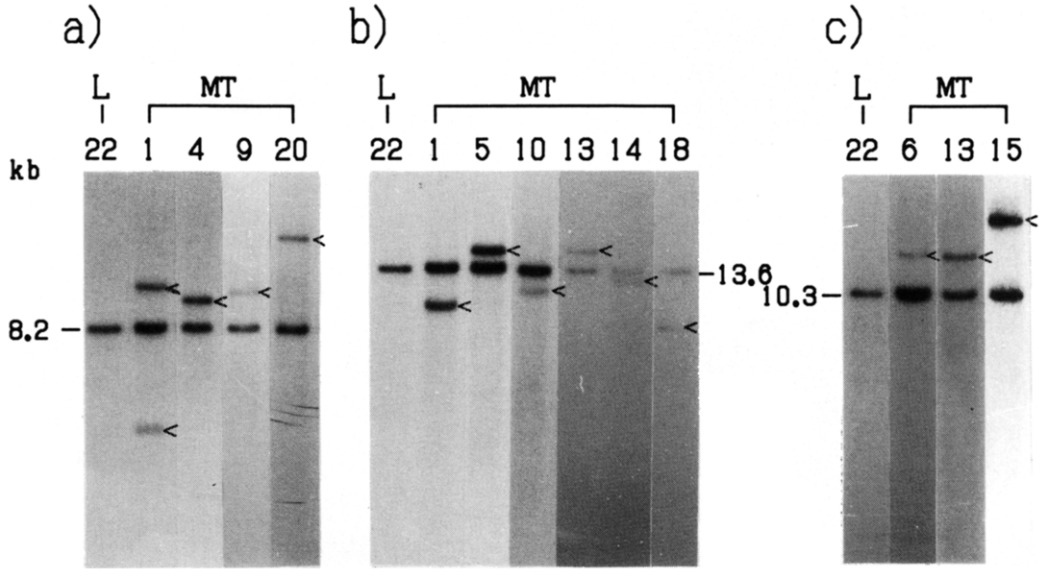

Fig. 3. Rearrangements of the int-1 *and* int-2 *regions in DNAs from SHN/S mouse mammary tumors (MT) and liver (L). Tumor and liver DNA were analyzed by using the combination of (a)* Eco*RI and* int-1*c, (b)* Bgl*II and* int-1*c, (c)* Eco*RI and* int-2*c probes. MT number is shown in Table* 1. *Arrows show rearranged bands by insertion of MMTV proviruses.*

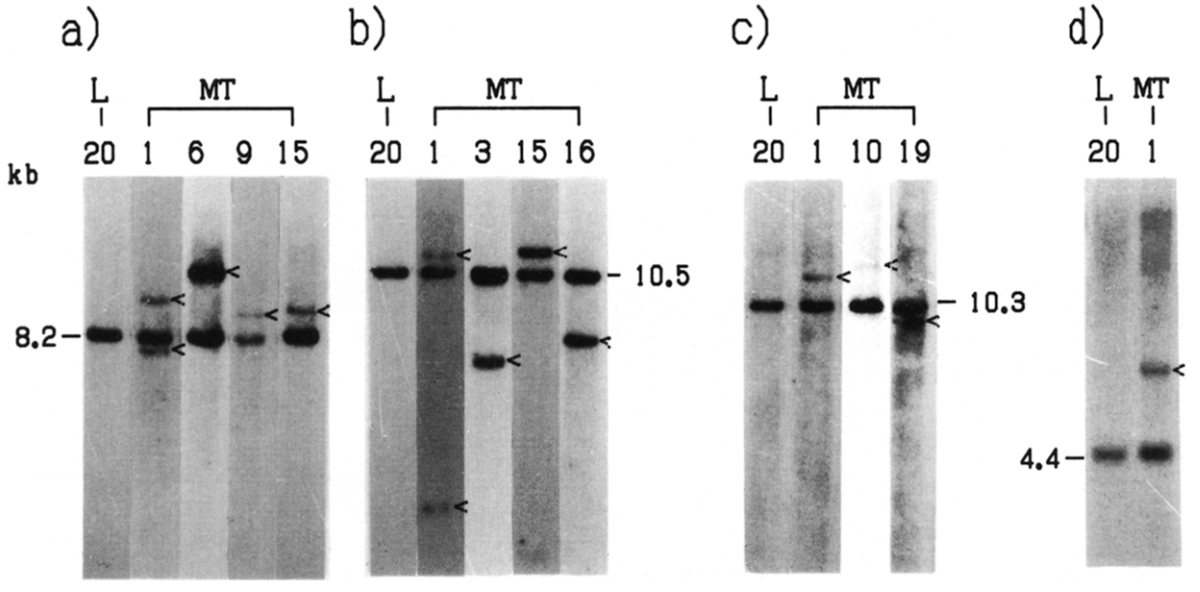

Fig. 4. Rearrangements of the int-1 *and* int-2 *regions in DNAs from SLN/S and SLN/Mei mouse mammary tumors (MT) and liver (L). Tumor and liver DNA were analyzed by using the combination of (a)* Eco*RI and* int-1*c, (b)* Bgl*II and* int-1*c, (c)* Eco*RI and* int-2*c, (d)* Kpn*I and* int-2*e probe. MT number is shown in Table* 1. *Arrows show rearranged bands by insertion of MMTV proviruses.*

Table 1. *DNA rearrangements of the* int-1 *and* int-2 *regions in SHN/S, SLN/S and SLN/Mei mouse mammary tumors*

Tissue	Sample No.	Mouse No.	Length of rearranged fragments (kb)				
			int-1c		*int*-2c	*int*-2c	*int*-2j
			*Eco*RI	*Bgl*II	*Eco*RI	*Kpn*I	*Eco*RI
Mammary tumor from SHN/S	1	601F	10.2 4.3	9.3	–	–	–
	2	601F	–	–	–	–	–
	3	601F	–	–	–	–	–
	4	593F	9.2	–	–	–	–
	5	594F	–	15.3	–	–	–
	6	602F	–	–	13.4	–	–
	7	602F	–	–	–	–	–
	8	602F	–	–	–	–	–
	9	638F	11.5	–	–	–	–
	10	631F	–	11.0	–	–	–
	11	631F	–	–	–	–	–
	12	631F	–	–	–	–	–
	13	630F	–	17.1	15.5	–	–
	14	630F	–	12.3	–	–	–
	15	615F	–	–	21.5	–	–
	16	615F	–	–	–	–	–
	17	623F	–	–	–	–	–
	18	623F	–	8.4	–	–	–
	19	623F	–	–	–	–	–
	20	623F	20.2	–	–	–	–
	21	629F	–	–	–	–	–
Mammary tumor from SLN/S	1	434F	11.6 7.7	13.1 2.6	14.3	7.2	–
	2	475F	–	–	–	–	–
	3	465F	–	5.4	–	–	–
	4	436F	–	–	–	–	–
	5	436F	–	–	–	–	–
	6	469F	14.5	–	–	–	–
	7	470F	–	–	–	–	–
	8	464F	–	–	–	–	–
	9	424F	11.1	–	–	–	–
	10	441F	–	–	15.8	–	–
	11	437F	–	–	–	–	–
Mammary tumor from SLN/Mei	12	Mei1	–	–	–	–	–
	13	Mei2	–	–	–	–	–
	14	Mei3	–	–	–	–	–
	15	Mei4	10.6	13.3	–	–	–
	16	Mei5	–	6.9	–	–	–
	17	Mei6	–	–	–	–	–
	18	Mei7	–	–	–	–	–
	19	Mei8	–	–	9.4	–	–

A minus (–) indicates that the tumor was negative for a novel restriction fragment.

and d) retained the rearrangements in the *int*-2 region. Namely, the frequency of MMTV provirus integration within either the *int*-1 and/or the *int*-2 region of SHN/S and SLN/S mammary tumor DNAs was 52% (11/21) and 45% (5/11), respectively. These results show that there was no significant difference in the frequency of MMTV integration into the *int* regions between the two strains. DNA from liver and eight mammary tumors of SLN/Mei mice were also analyzed. The frequency of provirus integration into either *int*-1 or *int*-2 was 38% (3/8) (Table 1, Fig. 4a, b and c). The numbers of endogenous MMTV proviruses and newly acquired proviruses in the tumors from SLN/Mei (data not

shown) were not significantly different from those from SLN/S.

DISCUSSION

MMTV endogenous provirus units that were transmitted by the germ-line are different and specific depending on the strain of mice [21–24]. Mtv-2 in GR [25, 26] and Mtv-4 in SHN [10] control the early development of mammary tumors. Whether SLN mice contain such a dominant gene has not been ascertained by foster-nursing and mating with a low mammary tumor strain of mice. However, SLN is supposed to contain the gene, since SLN harbored not only all restriction enzyme fragments contained in SHN, but also an additional provirus. Therefore, the difference in the age of onset of mammary tumors between the two strains could not be explained by the Mtv-4 gene, if the gene is a structural viral sequence of the germinal provirus. Genetic analysis of 32 markers showed that Hbb and Gpi-1 markers on chromosome 7 are the only differences between SHN and SLN mice [3]. This suggested that the strains preserved the same genetic background. In estimating the numbers of endogenous and exogenous MMTV provirus units, we did not consider that two *Eco*RI fragments could co-migrate, leading to different numbers of 5′ and 3′ ends. However, it is concluded from the band patterns shown in Fig. 2 that SLN mice contain relatively at least one more endogenous provirus unit than SHN. SLN mice may acquire an additional endogenous provirus, presumably during or after the separation of the strain. Alternatively, SHN mice may be a derivative of SLN from which this provirus has been lost. The additional provirus in SLN mice generated a 5′ 6.5 kb and a 3′ 5.1 kb *Eco*RI fragment. These fragments may be derived from Mtv-1, since the 5′ fragment has the same size as a 5′ fragment derived from Mtv-1 [24]. If the additional provirus is not unique to SLN mice, it is not unlikely to have been acquired by simple integration into the germ line after the strains were separated. To clarify these possibilities, the organization of endogeneous MMTV sequences have to be further investigated. Mtv-1 is known to be associated with late-occurring tumors and located on chromosome 7 [24, 27]. It is noteworthy that there are some differences in genetic loci located on chromosome 7 between SHN and SLN.

Both SLN and SHN contained common full-length or subgenomic MMTV proviruses except an additional provirus. These proviruses include presumably Mtv-8, Mtv-14 and Mtv-17, as they generate the same or similar sized of *Eco*RI fragments that the well-known Mtv units generate [24].

Mammary tumors from the SHN and SLN strains acquired one or more additional proviruses. Moreover, the frequency of MMTV proviral integration within the *int*-1 and *int*-2 regions of mouse mammary tumor DNA was 52% and 45% in SHN/S and SLN/S, respectively, and were not significantly different between the two strains. The high frequency of provirus integration within the *int* region can explain the high incidence of mammary tumors in the two strains, but not the difference in the age of onset of mammary tumors between them. The fact that approximately half the mammary tumors in the SHN/S and SLN/S strains did not integrate the proviruses within the *int* region suggests the presence of other unidentified integration sites or the involvement of other causative factors. We examined also the transcription of RNAs specific for the *int* regions (*int*-1c, -2c, -2e and *int*-3 [15]) and obtained a preliminary result that there was no significant difference in *int*-1 and *int*-2 between the two strains, and no detectable level of the *int*-3 transcript was found in both strains (data not shown).

SHN/S mice developed, frequently concurrently, two to four tumors per mouse, but SLN/S only one or rarely two tumors. The multiple mammary tumors developed in an individual mouse consisted of independent clones, because the integration numbers of proviruses, their restriction band patterns (data not shown) and the frequency of insertion within *int* regions were different depending on each tumor (Table 1). Similar results were obtained by Tanaka *et al.* who examined the origin of multiple mammary tumors developed in the same (SHN × C3H/He)F_1 mice by using two types of X-linked phosphoglycerate kinase isozymes as cellular mosaicism markers [28].

Mammary tumors can be induced by chemical carcinogens and hormones as well as MMTV. Highly tumorigenic hyperplastic alveolar nodules (HANs) induced by these carcinogens have been shown to have an exogenous MMTV provirus at a common site (*int*-H) in their DNAs [29]. The time of onset of HAN formation in SHN/S is earlier than that in SLN/S [4]. The proviral integration within the *int*-H region in both mammry tumors still remains to be solved.

Acknowledgements—We thank Dr H. Nagasawa for supplying the SLN/Mei mice bearing mammary tumors. We also thank Drs Y. Tsubura and N. Sarkar for helpful advice.

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