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Mouse Mammary Tumor Virus Integration Regions *int-1* and *int-2* Map on Different Mouse Chromosomes

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Two regions of mouse DNA which constitute common provirus integration sites in tumors induced by mouse mammary tumor virus have been identified and designated *int-1* and *int-2*. By examining a series of hamster-mouse somatic cell hybrids, we mapped the *int-2* locus to mouse chromosome 7 and confirmed the previous assignment of *int-1* to chromosome 15. This constitutes proof that *int-1* and *int-2* are discrete genetic loci. It is therefore possible that proviral activation of two distinct cellular genes may result in the same neoplastic disease.

Several lines of evidence have indicated that the DNA of normal somatic cells contains potential oncogenes which may be implicated in certain neoplastic diseases. The most comprehensive evidence both for the existence and the properties of such genes has come from the study of acutely oncogenic retroviruses in which cellular sequences (c-*onc*) have been transduced to become part of the viral genome (v-*onc*) (1). More recently, however, dominant transforming genes, in most cases related to known v-*onc* sequences, have been identified in the DNA of tumor cells with no known viral etiology (4, 26). In their normal context, such genes are presumably nononcogenic and require some perturbation or stimulus to become so. As a general rule, it would appear that this activation of a latent oncogene can be achieved either by enhanced expression of the normal gene product or structural alteration of the product, or perhaps both. Clearly both possibilities may pertain in the acutely oncogenic viruses in which the v-*onc* equivalents are expressed from viral transcriptional promoters and are frequently altered relative to their c-*onc* counterparts. In other situations, activation of the oncogene appears to be mediated by mechanisms such as point mutation (6, 19, 22, 23), chromosome translocation (20), or gene amplification (21). With these considerations in mind, it seems likely that nonacutely oncogenic retroviruses, whose genomes do not contain identifiable v-*onc* sequences, may also act via a cellular gene (25). Thus, whereas provirus integration may occur at essentially random sites in chromosomal DNA, in rare cells a provirus may become located adjacent to and consequently perturb the expression of a latent cellular oncogene. Direct evidence of this type of insertional mutagenesis has been well documented in tumors induced by avian leukosis viruses (7, 8, 17), but little is known about other nonacutely oncogenic retroviruses.

We have recently been examining provirus integration sites in tumors induced by mouse mammary tumor virus (MMTV), the B-type retrovirus associated with the high incidence of mammary carcinomas characteristic of certain inbred strains of mice. The interest in this virus stems from its unique tropism for the aleveolar cells of the mammary gland and the possibility that any potential c-*onc* gene involved in the malignancy may have correspondingly unique properties. The biology of the MMTV system and the progression of the disease are entirely consistent with the insertional mutagenesis model (2). Thus, the virus does not contain a v-*onc* equivalent, does not transform cells in culture, and causes tumors in animals only after prolonged latency. Tumors, when they arise, represent a rare event relative to the number of productively infected cells in the animal. They also appear to be clonal outgrowths derived from single phenotypically transformed cells and generally contain one or more newly integrated proviral elements as compared with normal tissue (3, 16, 18). By isolating recombinant DNA clones representing the junctions between chromosomal DNA and these acquired proviruses, we have prepared unique-sequence probes for the cellular DNA flanking the sites of integration. Using such probes, we have identified a defined region of chromosomal DNA in which an MMTV provirus has integrated in 19 out of 40 independent tumors examined to date (18). Although targeted integration of MMTV cannot be rigorously excluded, these findings suggest that MMTV may indeed exert its tumorigenic potential by altering the expression of a particular cellular gene.

In a parallel study, Nusse and Varmus have reported similar conclusions, implicating a specific chromosomal domain in MMTV-induced tumorigenesis (16). They have designated this provirus integration region MMTV *int-1* and mapped it to mouse chromosome 15 (16; R. Nusse and D. Cox, personal communication). However, at the structural level and as judged by cross hybridization of probes, the region we have identified, which we have termed *int-2*, shows no homology with the analogous *int-1* locus (18). These findings therefore suggest two possible conclusions: either that a single but extensive region (*int-1* and *int-2* each span about 25 kilobases [kb] of cellular DNA) may be involved and that the two studies have identified different parts of the same domain or that two distinct loci may contribute to the same neoplastic disease. To resolve this issue, we determined the chromosome assignment of *int-2*, as well as confirming that of *int-1*, and verified that they represent discrete loci occurring on different mouse chromosomes.

DNA from a series of somatic cell hybrids which had segregated different mouse chromosomes were analyzed by Southern blotting with probes specific for *int-1* or *int-2*. The cell hybrids were generated by fusion of Chinese hamster cells (E36) with peritoneal cells or spleen cells of BALB/c,

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A/J, and NFS *Akv-2* congenic mice. The characterization of these hybrids and their use in the chromosomal mapping of other cellular genes have been described elsewhere (10–13). A total of 26 hybrids were analyzed by using two different restriction enzymes to digest the cellular DNA before electrophoresis and blotting. In all cases, the conclusions drawn by using the two enzymes were in complete agreement.

The original characterization of the *int-2* locus was based on a unique 10-kb *Eco*RI fragment present in normal mouse DNA (Fig. 1 and reference 18). Under stringent conditions for washing the Southern blots (0.1× SSC [0.15 M NaCl plus 0.015 M sodium citrate] at 65°C), this 10-kb fragment does not appear to contain any repetitive sequence elements and can therefore be used directly as a hybridization probe. From the restriction map (Fig. 1), it is also clear that this single probe will recognize numerous characteristic fragments in normal mouse DNA, depending on the choice of restriction enzyme. Thus, the enzyme *Bam*HI, used in the present study, generates fragments of 6.2, 5.4, 3.0, and 2.8 kb, all of which react with this probe, although the homologous segment within the 6.2-kb fragment is very short and is therefore difficult to detect (see Fig. 2). In the hamster DNA common to all the somatic cell hybrids, the same probe recognizes a faint 3.0-kb *Bam*HI fragment under the conditions used. Conversely, the enzyme *Eco*RI generates the expected 10-kb fragment in mouse DNA and a weakly cross-reacting 8.0-kb species in hamster DNA (Fig. 2).

Figure 2 shows examples of the results obtained with a number of the hybrid cell DNAs. On the basis of this type of data, all the hybrids were scored as positive or negative for the presence of *int-2*. Examination of the mouse chromosome content of 21 of these hybrids showed that all those which were positive for *int-2* also carried mouse chromosome 7, whereas the negative hybrids did not (Table 1). Furthermore, hybrids which contained chromosome 7, but at low frequency (e.g., HM20 in Fig. 2), showed weak hybridization signals on the Southern blots. One of the positive hybrids contained chromosome 7 as its only mouse genetic material. In contrast, all other mouse chromosomes displayed discordant segregation with *int-2* (Table 1). Finally, five additional hybrids, which were not karyotyped, were typed for isoenzyme markers on 14 mouse chromosomes. Three of these hybrids contained *int-2* and chromosome 7, whereas the remaining two lacked both.

Although the *int-1* locus was previously mapped to mouse chromosome 15 (R. Nusse and D. Cox, personal communication), we decided to verify this point with the hybrids used in the present study. The nitrocellulose filters used to locate *int-2* specific sequences were therefore rinsed in alkali to remove residual radioactivity and hybridized to an *int-1* specific probe provided by R. Nusse. A clear correlation between the presence of *int-1* and chromosome 15 was observed (Table 2). Of the 17 hybrids which were positive for the presence of *int-1*, all but one of them retained an

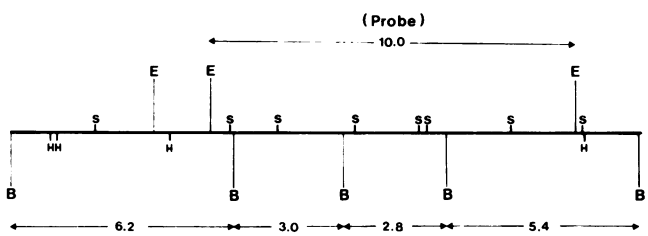

FIG. 1. Partial restriction map of the MMTV *int-2* locus. A detailed restriction map spanning 25 kb of the *int-2* region will be presented elsewhere. The figure shows an abridged version in which the cleavage sites for the enzymes *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), and *Sac*I (S) are indicated. The numbers refer to the sizes in kb of fragments generated by *Eco*RI or *Bam*HI which would be detectable by using the 10-kb *Eco*RI fragment as a probe.

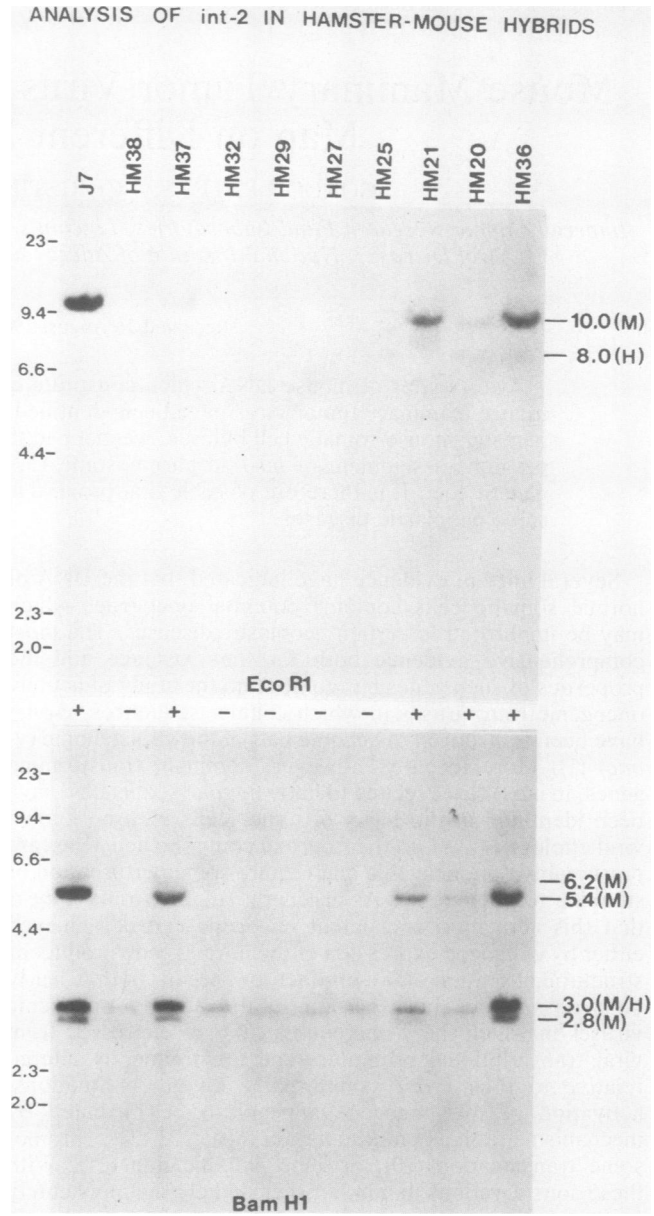

FIG 2. Hybridization of an *int-2* probe to DNA from a series of hamster-mouse hybrid cells. High-molecular-weight DNA (10 μg) from a series of 26 hamster-mouse hybrids was digested with either *Eco*RI or *Bam*HI, fractionated by electrophoresis through 1% agarose gels, and transferred to nitrocellulose by standard procedures (14, 18). The filters were hybridized with a probe corresponding to the 10-kb *Eco*RI fragment from *int-2* (Fig. 1), nick translated with [α-^{32}P]dCTP to a specific activity of ca. 10^8 cpm/μg of DNA (18). Hybridization was carried out in 50% formamide at 42°C as described (18), and filters were then washed in 0.1× SSC–0.1% sodium dodecyl sulfate at 65°C for 1 h before autoradiography. Numbers on the left of each panel refer to the sizes in kb of the *Hin*dIII-cleaved λ DNA fragments used as standards. Numbers on the right indicate the fragments observed in mouse (M) or hamster (H) DNA.

TABLE 1. Correlation of mouse chromosomes and MMTV *int-2* in 21 mouse-hamster hybrids

Mouse chromosome	No. of hybrid clones				% Discordant
	+/+[a]	−/−	+/−	−/+	
1	7	3	8	3	52[b]
2	7	5	8	1	42
3	5	3	10	3	62
4	3	5	12	1	62
5	1	5	14	1	71
6	7	5	8	1	42
7	15	6	0	0	0
8	4	6	11	0	52
9	6	6	9	0	42
10	4	6	11	0	52
11	0	6	15	0	71
12	11	5	4	1	24
13	5	5	10	1	52
14	3	6	12	0	57
15	12	4	3	2	24
16	4	4	11	2	62
17	9	2	6	4	48
18	7	5	8	1	43
19	6	5	9	1	48
X	5	4	10	2	57

[a] Presence of *int-2*/chromosome retention.

[b] Seven hybrids contained *int-2* and chromosome 1; three hybrids lacked both *int-2* and chromosome 1; 11 hybrids contained either *int-2* or chromosome 1.

TABLE 2. Correlation of mouse chromosomes and MMTV *int-1* in 20 mouse-hamster hybrids

Mouse chromosome	No. of hybrid clones				% Discordant
	+/+[a]	−/−	+/−	−/+	
1	7	3	10	0	50
2	8	2	9	1	50
3	5	3	12	0	60
4	4	3	13	0	65
5	2	2	15	1	80
6	6	2	11	1	60
7	14	2	3	1	20
8	3	3	14	0	70
9	5	3	12	0	60
10	4	3	13	0	65
11	0	3	17	0	85
12	13	2	4	1	25
13	5	2	12	1	65
14	2	2	15	1	80
15	16	3	1	0	5
16	5	1	12	2	70
17	7	2	10	1	55
18	6	1	11	2	65
19	8	3	9	0	45
X	6	2	11	1	60

[a] Presence of *int-1*/chromosome retention.

intact chromosome 15. The one exceptional hybrid did not contain a recognizable chromosome 15 as judged by karyotype analysis, nor could any translocation chromosomes be identified by staining with Hoechst 33258, which specifically stains mouse centromeric DNA. However, at least a fragment of mouse chromosome 15 must have been retained in this hybrid because it could be detected by hybridization with a mouse c-*myc* probe (5; P. N. Tsichlis, P. G. Strauss, and C. A. Kozak, submitted for publication). Since c-*myc* is at the distal end of chromosome 15, these data suggest that *int-1* is at the same end of the chromosome.

The possibility of homology between the MMTV integration regions and known cellular oncogenes is clearly an important issue. Recently, both the ras_1^H and *fes* oncogenes have also been mapped to mouse chromosome 7, prompting us to examine their relationship, if any, to *int-2*. However, by cross hybridization of probes at reduced stringency, we were unable to detect any homology between the *int-2*, ras_1^H, and *fes* sequences. These data suggest that *int-2* represents a unique sequence on chromosome 7 which may be involved in oncogenic transformation.

As defined on the basis of provirus integration sites, the *int-2* locus spans at least 25 kb of cellular DNA. Within this region, the exact location of the relevant provirus in each tumor is quite variable, consistent with random integration at the nucleotide level (18). However, the data cannot formally exclude the possibility that the *int-2* domain as a whole represents a region of chromatin which is particularly favorable for MMTV provirus integration. For example, some inbred strains of mice carry an endogenous MMTV provirus on chromosome 7 at the *Mtv-1* locus (9, 24), suggesting that these germline sequences may also have been targeted to the same region of chromosomal DNA. This site would presumably have been unoccupied in the mice used in characterizing *int-2* since neither the BALB/c nor the BR6 strain contains an endogenous provirus equivalent to *Mtv-1* (18). To establish that *int-2* and *Mtv-1* are indeed distinct loci, we analyzed the integrity of the *int-2* region in uninfected tissue from C3H mice, which carries *Mtv-1* (24), and confirmed that these mice contain an intact *int-2* locus, at least over a range of 25 kb. Furthermore, integration sites for endogenous and exogenously acquired MMTV proviruses have now been located on several different mouse chromosomes, (9, 15, 24), suggesting that targeted integration is unlikely to account for the phenomenon observed in the *int-1* and *int-2* regions.

Thus, two regions of mouse DNA have now been identified which may contain genes involved in mammary carcinogenesis, since integration of an MMTV provirus in one or other of these domains has been shown to occur in a high percentage of virally induced mammary tumors. The present study has confirmed that these are quite independent regions since they are located on different chromosomes and show no cross homology, raising the possibility that two (or more) distinct cellular genes may contribute to the same neoplastic disease. The structure and expression of RNA transcripts derived from these integration regions is presently under investigation.

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