Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15

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Most mammary carcinomas induced in C3H mice by the mouse mammary tumour virus (MMTV) bear a new proviral insertion within a highly conserved locus on chromosome 15 called int-*1. A transcriptional unit within this locus is inactive in all tested normal tissues but expressed at low levels in mammary tumours with proviral insertions positioned on either the 5′ and 3′ sides of the gene. Transcription of the proviruses proceeds away from* int-*1; thus an indirect mechanism appears to activate expression of this putative oncogene.*

THE proviruses of retroviruses can integrate at many sites in host genomes; thus, like other movable genetic elements, they act as insertion mutagens[1]. Two classes of proviral insertion mutations have been described: recessive mutations that disrupt and prevent expression of genes[2–6] and presumably dominant mutations that activate gene expression, leading to induction of tumours by retroviruses without their own oncogenes[7–13]. For example, during the induction of B-cell lymphoma by the avian leukosis virus (ALV), proviral insertion mutations augment transcription of c-*myc*, the cellular progenitor of a retroviral oncogene[7], either by provision of a retroviral promoter[7–10] or by an indirect effect on a host promoter[10].

The proposal that retroviruses lacking oncogenes induce tumours by insertion mutation of host genes suggests a strategy for the isolation of novel oncogenes. By molecular cloning of a single new mouse mammary tumour virus (MMTV) provirus from a C3H mouse mammary tumour, we have isolated a region of the mouse genome that contains an MMTV proviral insertion in the majority of C3H mammary tumours[13]. Although this region lacks homology with any of the available retroviral oncogenes, we have previously argued that it harbours an oncogene, called *int*-1, instrumental in mammary tumorigenesis because (1) MMTV proviruses can integrate at many sites in the host genome (implying that insertions near *int*-1 confer a selective growth advantage) and (2) because the insertions are accompanied by the appearance of transcriptional products from *int*-1, a silent domain in non-neoplastic mammary tissue.

We demonstrate here that insertion mutations in the *int*-1 locus usually stimulate transcriptional activity by an indirect mechanism: MMTV proviruses are located either upstream from transcribed *int*-1 sequences in the opposite orientation or downstream in the same orientation. We also assign the *int*-1 gene to mouse chromosome 15 and show that *int*-1, like the progenitors of viral oncogenes, has been conserved during evolution.

Locating and orienting MMTV proviruses

We reported previously[13] that 18 of 26 C3H mouse mammary tumours contain MMTV proviral DNA within a common region of approximately 20 kilobases (kb). The insertions are distributed with roughly equal frequency on both sides of a portion of the locus that is represented in tumour-specific, 2.6-kb polyadenylated RNA. To assess the mechanisms involved in the activation of the *int*-1 transcriptional unit, we have now determined the sites of proviral insertion more precisely and ascertained the transcriptional directions of the proviruses and the *int*-1 gene. These studies involved physical mapping of the interrupted *int*-1 loci with restriction endonucleases, using the DNA transfer method and a collection of molecular annealing reagents specific for portions of *int*-1 and for the 5′ and 3′ halves of MMTV proviral DNA. The *int*-1 probes were derived by plasmid subcloning of unique sequence DNA from recombinant λ bacteriophages containing overlapping inserts of the *int*-1 domain from a library of normal BALB/c mouse DNA. Probe C detects *int*-1 RNA in mammary tumours; probe D represents a region located leftward of probe C on the *int*-1 map as it is conventionally drawn (see Fig. 3). The probes used to orientate proviral DNA [MMTV-*env* and MMTV-*gag* (Fig. 1)] represent portions of MMTV DNA that reside near the 5′ and 3′ ends of the provirus but exclude sequences from the long terminal repeats (LTRs). In general, our strategy was to use enzymes that cleaved *int*-1 at widely separated sites and divided MMTV proviral DNA into two portions distinguishable with MMTV-*env* and MMTV-*gag* probes.

Proviruses on the 5′ side of *int*-1

Two enzymes, *Bgl*II and *Kpn*I, proved to be particularly useful for the analysis of MMTV proviruses present to the left of the *int*-1 transcriptional unit. (We will show below that transcription of *int*-1 proceeds from left to right on the physical map; thus, proviruses inserted to the left of the region represented by probe C can be considered to lie 5′ to at least a portion of the transcriptional template.) *Bgl*II cleaves the normal C3H mouse DNA at a leftward site (position −21 on the map in Fig. 3) to generate a 14-kb fragment that anneals with probe C. In addition, *Bgl*II cleaves the MMTV provirus at two sites, one of them near the middle of the element (Fig. 1). Proviral DNA in eight tumours was found to disrupt the normal 14-kb *Bgl*II fragment from one of the two *int*-1 alleles. As illustrated for six of these tumours in Fig. 1*a* and *b*, the novel fragments also annealed with the MMTV-*gag* probe, indicating that the proviruses are positioned either to the right of the probe C homology in the same transcriptional orientation as *int*-1 or to the left of the probe C region in the opposite orientation. In all eight tumours, additional mapping, illustrated here for a few tumours with *Kpn*I and probe D, showed that the latter possibility was correct (Fig. 1*c*, *d*). *Kpn*I cleaves the *int*-1 domain at the most leftward site we have mapped (position −23) and at position −11, to generate a fragment of 12 kb that hybridizes with probe D. The MMTV provirus is cut once by *Kpn*I. Novel *Kpn*I fragments annealing with probe D (Fig. 1*c*) were also detected by the MMTV-*env* probe (Fig. 1*d*), demonstrating that the proviruses are inserted upstream from the probe C homology region in orientations opposite to the transcriptional direction of *int*-1.

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Table 1 Segregation of mouse *int*-1 and mouse chromosome in Chinese hamster–mouse somatic cell hybrids

Chromosome	Marker enzyme	*Int*-1/chromosome (no. of clones) +/+	+/−	−/+	−/−	% Discordance
1	PEP-3	5	4	1	2	42
2	AK-1	15	2	8	4	34
3	—	2	7	1	2	67
4	PGD	13	5	4	8	30
5	—	2	7	1	2	67
6	TPI-1	3	6	7	5	62
7	GPI-1	4	5	2	1	58
8	GR-1	4	5	1	2	50
9	MOD-1	1	8	1	2	75
10	PEP-2	2	7	2	1	75
11	—	0	9	0	3	75
12	ACP-1	5	4	1	2	42
13	—	4	5	0	3	42
14	NP-1	10	6	5	7	39
15	AS-2	17	0	1	10	4
16	SOD-1	3	6	2	0	73
17	GLO-1	2	7	1	2	67
18	PEP-1	7	2	5	8	32
19	GOT-1	12	6	1	11	23
X	HPRT	15	3	12	0	50
Y	—	0	9	0	3	75

Thirty clones of hybrids formed by polyethylene glycol fusion of mouse spleen cells or macrophages with an established Chinese hamster cell line (380-6), deficient for hypoxanthine phosphoribosyl transferase (HPRT), were analysed for mouse *int*-1 and enzyme markers, although not all clones were analysed for every enzyme marker. Twelve of the hybrid clones were also analysed by karyotype analysis as decribed previously[22]. The symbols for the marker enzymes, their chromosome assignments and the electrophoretic procedures used to separate the Chinese hamster and mouse enzymes have been previously described[22]. In general, the presence or absence of a given mouse chromosome, as determined by karyotypic analysis, agreed with the presence or absence, respectively, of the enzyme marker for that chromosome. However, in several instances, the enzyme marker for a given chromosome was present in a clone that karyotypically lacked that mouse chromosome, indicating chromosome breakage and/or rearrangement. The presence of mouse chromosomes 3, 5, 11, 13 and Y was based on karyotype analysis alone.

Proviruses on the 3′ side of *int*-1

Similar analytical strategies were applied to DNA from 10 tumours previously shown to have insertions between positions 0 and −8 on the *int*-1 map; an 11th tumour (number 24), previously thought to lack an *int*-1 insertion, was also found to have a provirus within this region during the extended tests. Comparative annealings with *int*-1 and MMTV probes (ref. 13 and data not shown) revealed that all 11 proviruses were oriented in the same direction, with transcription proceeding from left to right on the *int*-1 map.

A particularly interesting part of this analysis, pertinent to the mechanism of expression of *int*-1, is illustrated in Fig. 2. When DNA from tumour 53 was cleaved with *Bam*HI and annealed with probe C, two novel fragments were detected in digests of each sample, indicating that the insert is positioned within the region detected by the probe. Similar results were obtained with DNA from tumour 11 (not shown). Proviruses in three other tumours (numbers 55, 12 and 15) were found to be positioned at very similar sites, about 0.6–0.8 kb to the right of the insertion site in tumour 53 (Fig. 2 and unpublished data). The inserts in these tumours lie within the transcriptional unit, and affect the size of resulting transcripts (see below).

We conclude that 19 of 26 C3H mammary tumours carry MMTV proviruses in the *int*-1 locus (Fig. 3). Eight proviruses are distributed between positions −11 and −18; each of these is oriented so that transcription of the provirus proceeds from right to left. Eleven proviruses are located within the region extending from position −8 to 0, and all are oriented so that transcription proceeds from left to right. Mapping with several endonucleases provides no evidence for deletions within either proviral DNA or *int*-1; the several atypical restriction maps of proviral DNA we encountered are best explained by restriction site polymorphisms (data not shown).

int-1 transcripts

We have previously shown that tumours bearing proviruses in the *int*-1 region contain polyadenylated transcripts that anneal with probe C, whereas no RNA homologous to probe C is detectable in normal mammary glands[13]. The abundance of the *int*-1 transcripts is not great; less than 10 copies per cell have been estimated by three independent methods (unpublished results).

To judge the mechanism by which the insertion mutations activate expression of *int*-1, it was necessary to determine the direction of transcription of the cellular gene. The *Bam*HI–*Eco*RI fragment that constitutes probe C was recloned in both orientations in the bacteriophage vector M13. Restriction mapping of the replicative forms established the orientation of each insert and hence the polarity of strands present in single-stranded virion DNA (data not shown). Radioactive probes were synthesized by random priming on templates provided by two phages, each bearing one of the two strands. Only one of these probes was found to anneal to RNA from tumour 44, previously shown to contain an *int*-1 transcript 2.6 kb in length (Fig. 4*a*); the composition of the probe established the direction of transcription to be from left to right on the *int*-1 map as presented in Fig. 3.

The *int*-1 transcripts characterized in our earlier report were, like those in tumour 44, approximately 2.6 kb long[13]. In a wider survey of all those tumours from which undegraded polyadenylated RNA could be obtained, we discovered atypical *int*-1 transcripts of 3.2 kb in tumour 11 and of 3.8 kb in tumours 12 and 15 (Fig. 4*b*). In these three tumours, no 2.6-kb *int*-1 RNA was found. The conventional 2.6 kb *int*-1 RNA species was present in all the other tested tumours with *int*-1 insertions (for example, in tumours 24 and 13 in Fig. 4*b*) and in one tumour (number 14) in which an *int*-1 mutation has not been found (unpublished data).

The probable composition of the larger, atypical RNA species was suggested by an intriguing correlation with the site of insertion: in tumours 11, 12 and 15, integration of proviral DNA occurred within or near the right-hand boundary of the region represented by probe C (see Fig. 2). (Suitable RNA samples were not available from the other tumours, 53 and 55, with insertions in this region.) If the proviruses were located within the transcribed domain, upstream from the natural polyadenylation site for *int*-1 RNA, the 5′ LTR would be appropriately positioned to provide a surrogate polyadenylation site. The enlarged size of the *int*-1 transcript could then be ascribed to the length of the U3 region of the MMTV LTR (~1.2 kb)[14–16]. A similar situation has been encountered in an avian leukosis virus (ALV) insertion mutant affecting the chicken c-*myc* locus[10].

It was not possible to test this explanation by attempting to anneal a labelled probe for the MMTV LTR directly to gel fractionated RNA, because virus-specific RNA transcribed in a normal fashion from proviral DNA was vastly more abundant than transcripts from *int*-1. We attempted to circumvent this problem by using the 'sandwich hybridization' procedure[10,16] to test for RNA molecules containing MMTV U3 sequences covalently linked to *int*-1 sequences. Restriction fragments of phage DNA containing a portion of the *int*-1 domain were fractionated in an agarose gel, transferred to nitrocellulose filters, and annealed with unlabelled RNA from tumour 12 (containing a 3.2 kb transcript) or from tumour 13 (with a conventional 2.6 kb transcript). After removal of unannealed RNA, the filter was incubated with a ^{32}P-labelled *Pst*I fragment containing virtually all of the MMTV LTR. One autoradio-

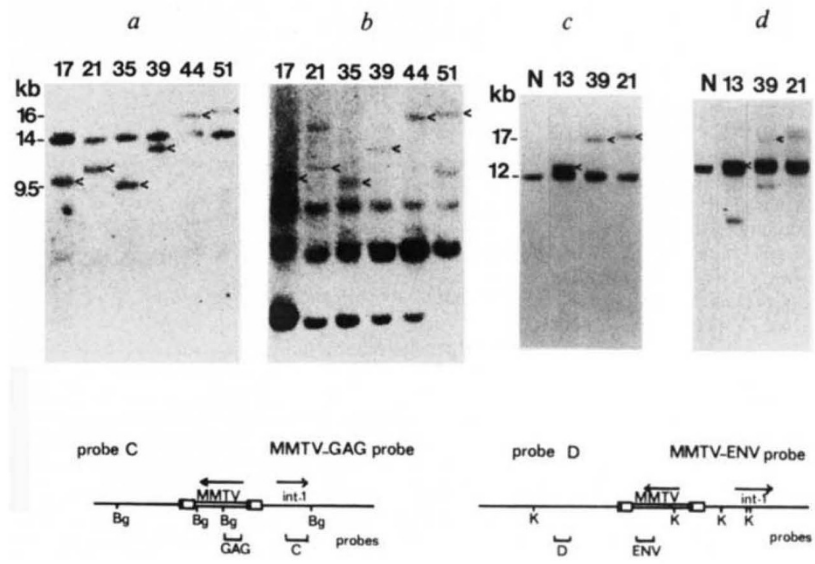

Fig. 1 MMTV proviruses on the 5′ side of the *int*-1 transcriptional unit are in the transcriptional orientation opposite to that of *int*-1. DNA samples (10 μg) from mammary tumours (numbers indicated on top) were digested with *Bgl*II (Bg, panels *a* and *b*) or *Kpn*I (K, panels *c* and *d*), electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose[13]. The filters were first incubated with probe C (*a*) or probe D (*c*), washed and exposed to X-ray film. Subsequently, the probes were removed from the filters and the filters were incubated with MMTV-*gag* probe (*b*) or with MMTV-*env* probe (*d*). The origin of the probes is indicated in the diagrams at the bottom of the figure and in Fig. 3; MMTV-*gag* (3.2 kb) is a *Pvu*II–*Bgl*II fragment obtained from a clone of proviral DNA endogenous to GR mice (ref. 56); MMTV-*env* (1.8 kb) is a *Pst*I fragment derived from MMTV (C3H) DNA and cloned into pBR322 (ref. 57). Arrows indicate tumour-specific bands that show rearrangements of the *int*-1 locus detected with probes C and D and bands in the same position detected with the MMTV probes.

graphic signal, at the position of the *int*-1 fragment homologous to probe C, was observed in the experiment conducted with RNA from tumour 12; no reaction was detected with the sample from tumour 13 (Fig. 4*c*). From this experiment we conclude that *int*-1 and MMTV sequences are covalently joined in transcripts from tumour 12; we presume, but have not proved, that the MMTV sequences are located at the 3′ end of the 3.2-kb transcript in this tumour.

Conservation of the *int*-1 locus

We have proposed that the *int*-1 domain harbours an oncogene that contributes to mammary tumorigenesis following transcriptional activation by proviral integration. However, the locus lacks homology to all tested retroviral oncogenes including *sis*, *ski*, *fos*, *fms* and *yes* (data not shown), as well as the 11 examined previously[13], and it does not anneal with the NIH/3T3 cell transforming gene detected in mammary tumour DNA by Lane *et al.* (ref. 17 and our unpublished results with M. A. Lane and G. M. Cooper). Moreover, we have been unable to measure *int*-1 RNA in a variety of tissues from mature mice (liver, spleen or brain), in cultured mouse cells, or in normal mammary glands from pregnant or lactating females (data not shown). Since no protein product of *int*-1 has been identified, we sought support for the idea that the *int*-1 region contains a coding domain by testing for conserved *int*-1 sequences, homologous to probe C, in the DNA of other vertebrates. As illustrated in Fig. 5, *Bam*HI fragments that anneal effectively with probe C in conditions of only moderately reduced stringency are present in digests of DNA from fish, birds and several mammals, including man. These results are similar to those observed with conserved cellular genes, including the known oncogenes[18]. In addition, as found for the progenitors of some retroviral oncogenes[19], sequences homologous to *int*-1 can be detected in the genome of *Drosophila melanogaster* (lane 1).

Mouse chromosome 15

Specific karyotypic anomalies, some involving putative oncogenes, have recently been found to be associated with a number of different neoplasms[20]. Trisomy of mouse chromosome 13, for example, is frequently observed in MMTV-induced mammary tumours[21]. It was therefore of interest to determine the chromosomal location of *int*-1. To assign *int*-1 to a specific mouse chromosome, we used probe C to detect restriction fragments containing *int*-1 in digests of genomic DNA from 30 Chinese hamster–mouse somatic cell hybrid clones segregating

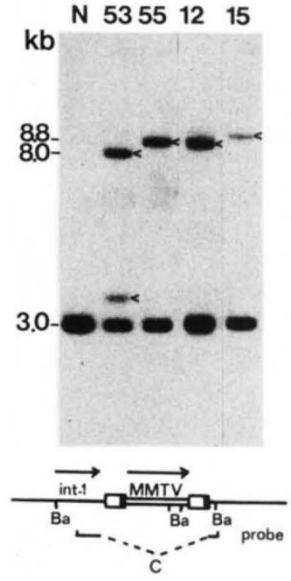

Fig. 2 Proviruses on the 3′ side of *int*-1 are in the same transcriptional orientation as *int*-1. Tumour DNA (number indicated on top, N: normal DNA) was digested with *Bam*HI (Ba), transferred to nitrocellulose filters and annealed with probe C. The sizes of the arrowed, novel restriction fragments and additional mapping data (not shown) show that the proviruses were integrated as drawn at the bottom of the figure. This illustration shows the provirus from tumour 53, providing an explanation for the two novel *int*-1 fragments detected with probe C. Proviruses in the other tumours are located further rightward (see Fig. 3).

mouse chromosomes. These hybrids together contain the full complement of murine chromosomes except for chromosomes 11 and Y (ref. 22). The mouse chromosome complement of each hybrid clone was determined by isozyme and/or karyotype analyses as previously described[22]. The single *int*-1-specific *Eco*RI fragment of 8.6 kb present in digests of genomic mouse DNA could be easily distinguished from the *int*-1-specific *Eco*RI fragment of 2.8 kb derived from Chinese hamster DNA (not shown). An analysis of the segregation of murine *int*-1 and enzyme markers for each of 16 mouse chromosomes (all but 3, 5, 11, 13 and Y) revealed 96% concordant segregation of *int*-1 and arylsulphatase A (AS-1), an enzyme marker for mouse chromosome 15, but discordant segregation of *int*-1 and the

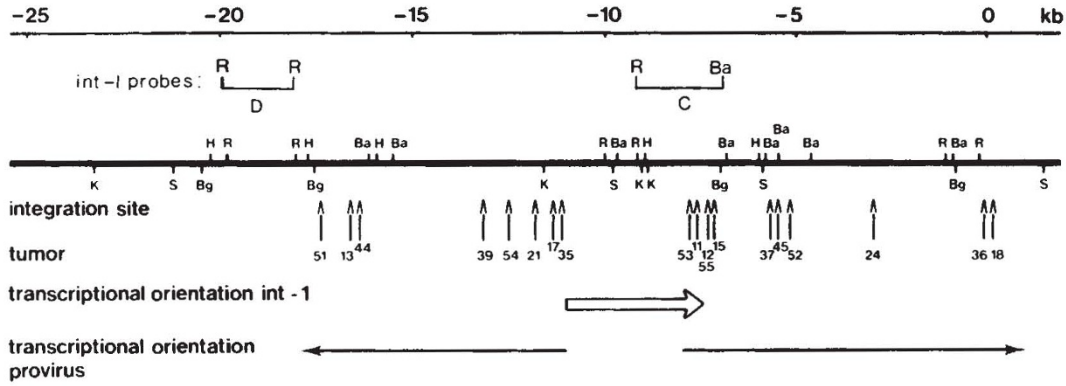

Fig. 3 A map of the proviral integration sites in the *int*-1 locus in C3H mice. Restriction enzyme sites were mapped using C3H and BALB/c chromosomal DNA and DNA from several clones of recombinant bacteriophage containing overlapping inserts from normal BALB/c DNA. Position 0 is defined as the insertion site of the MMTV provirus in tumour 18; this provirus was originally cloned with its flanking DNA to obtain DNA from the *int*-1 locus. Other molecular clones, encompassing positions −22 to +20 on the *int*-1 map, were obtained by sequential screening of a Charon 4A bacteriophage library of normal BALB/c mouse DNA with probes from the *int*-1 region, as described previously[13]. R, *Eco*RI; Ba, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; S, *Sac*I; H, *Hin*dIII. Probes C and D are fragments of single copy DNA positioned as indicated on the map. Vertical arrows above tumour numbers designate sites of integration. The wide horizontal arrow denotes transcriptional orientation of *int*-1 and the approximate extent of the transcribed domain. The narrow horizontal arrows show the direction of transcription of proviruses integrated in the regions demarcated by the arrows.

other enzyme markers (Table 1). When probe C was removed from the DNA blots and the samples reannealed with a probe for the c-*myc* oncogene (data not shown), we observed perfect concordance between *int*-1 and the mouse *Eco*RI DNA fragment derived from the c-*myc* locus previously assigned to mouse chromosome 15 (refs 23, 24). This finding, consistent with the isoenzyme results, provided further evidence for the assignment of *int*-1 to mouse chromosome 15. Karyotype analyses of 12 selected hybrid clones ruled out an association between *int*-1 and chromosomes 3, 5, 11 13 or Y and were also consistent with the assignment of *int*-1 to chromosome 15 (Table 1). The single discordant clone, which was weakly positive for AS-2 but negative for *int*-1, revealed no evidence of a recognizable mouse chromosome 15. Presumably, this hybrid contains a small, non-recognizable fragment of chromosome 15 which includes AS-2 but not *int*-1. Taken together, these data indicate that *int*-1 is located on mouse chromosome 15.

Implications

The results described here support our previous contention that tumour induction by MMTV involves insertion mutations that activate a previously unknown gene called *int*-1. At the same time they promote confidence in the general proposition that oncogenic viruses lacking viral oncogenes may initiate tumour growth by insertional mechanisms. This hypothesis fosters an experimental strategy for isolation of oncogenes that is formally analogous to what has been termed 'transposon tagging' in other systems[25]: identification of the mutagenic provirus in tumour DNA should lead directly to the isolation of a linked, mutated cellular oncogene. These ideas now receive support from studies of avian B-cell lymphomas induced by at least two different retroviruses[7,11], erythroleukaemia induced by an avian virus that also causes B-cell lymphoma[26], T-cell lymphoma induced by murine leukaemia virus[27] and mammary tumours induced by MMTV[13,28]. At present only circumstantial evidence implicates the affected cellular genes in the oncogenic process: (1) mutation of the same gene in several tumours of the same type; (2) augmented expression and (3) in some cases homology to progenitors of retroviral oncogenes.

Application of the transposon tagging method has led to the discovery of at least two potential mammary oncogenes: the *int*-1 gene commonly implicated in tumours arising in C3H mice and the *int*-2 domain recently found by Peters *et al.*[28] to be interrupted by proviral insertions in about half of the mammary tumours arising in the BR6 mouse strain. These two targets lack evidence of homology with known retroviral oncogenes or with each other, and they are located on different chromosomes (C. Dickson and R. N., unpublished results). In addition, since substantial numbers of tumours lack insertions in either domain, there may be yet other targets for MMTV insertion mutations.

The case for oncogenic roles for *int*-1 and *int*-2 rests primarily on the argument that MMTV proviral DNA appears capable of integration into many different regions in the mouse genome; hence the repeated finding of proviruses at these two loci implies that the tumours are clonal outgrowths of cells[29] with a growth advantage conferred at least in part by the insertion mutations. The case for *int*-1 also rests on the evidence that the locus is transcriptionally active in tumours bearing *int*-1 mutations but not in normal tissues. Although a protein product of *int*-1 has not been identified, a coding function is implied by the conservation of at least part of the transcribed domain during vertebrate evolution (Fig. 5); these findings conform to those obtained with the cellular genes accepted as potential oncogenes by virtue of their homology with retroviral oncogenes.

Our assignment of *int*-1 to mouse chromosome 15 does not offer additional insight into its role as an oncogene: karyotypic abnormalities involving this chromosome have not been reported in mouse mammary tumours, and no endogenous MMTV proviruses have been assigned to chromosome 15 (ref. 30). However, an unrelated oncogene, c-*myc*, also present on mouse chromosome 15, recombines with an immunoglobulin locus on chromosome 12 to produce the translocations found in several plasmacytoma lines[24,31]. Although the distance between *int*-1 and c-*myc* on mouse chromosome 15 is not known, mammary tumours with proviral insertions at *int*-1 do not display abnormal expression of c-*myc* (unpublished data of authors).

The core of the present report concerns the mechanisms by which MMTV proviruses activate expression of *int*-1. In ALV-induced bursal lymphomas, augmented expression of c-*myc* is most commonly achieved by a mechanism referred to as promoter insertion: ALV proviral DNA on the 5′ side of c-*myc* exons in the same transcriptional orientation provides an efficient transcriptional promoter in the form of an LTR[7–10]. However, in the 19 MMTV-induced mammary tumours studied here we have found no instance in which a promoter insertion mechanism activates expression of *int*-1. Instead, MMTV proviruses are positioned either on the 5′ side of the transcriptional template for *int*-1 in the opposite orientation or on the 3′ side in the same orientation. (This rule, however, is not inviolable: in one tumour recently obtained from a BALB/cfC3H mouse, a provirus is present on the 5′ side of the *int*-1 transcriptional domain in the same orientation (R.N., unpublished.)

Previous examples of the two arrangements observed here, in which an LTR cannot serve as promoter for an activated gene, have been described in ALV-induced bursal lymphomas[10]. In these few instances, the effect on expression of c-*myc* was ascribed to a retroviral 'enhancer' element, akin to similar elements first identified in papovavirus genomes[32–38]. Using

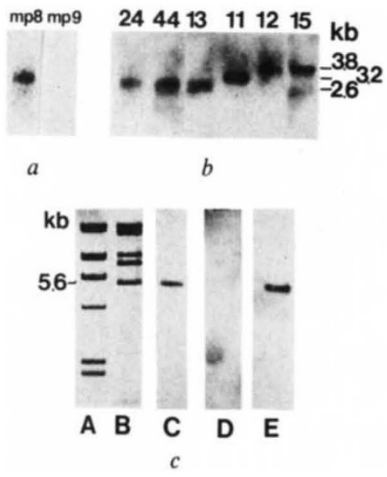

Fig. 4 Analysis of *int*-1 transcription in mammary tumours. *a*, The direction of transcription. 5 μg of polyadenylated RNA from mammary tumour 44 was subjected to electrophoresis in a 1% agarose gel with 2.2 M formaldehyde, transferred to nitrocellulose filters and hybridized with a strand-specific radioactive probe derived by subcloning probe C (a *Bam*HI–*Eco*RI fragment) in the single-stranded bacteriophage derivatives of M13, mp8 and mp9, in both orientations. The autoradiogram shows the hybridization to 2.6-kb RNA with probes made by transcribing the recombinant M13mp8 DNA with reverse transcriptase in the presence of ^{32}P-dCTP with oligomers of calf thymus DNA as random primers. A probe made similarly from the recombinant M13mp9 DNA did not detect a transcript. The direction of transcription of the *Eco*RI–*Bam*HI fragment must proceed from the *Eco*RI site to the *Bam*HI site or from left to right on the *int*-1 map as shown in Fig. 1. *b*, The length of *int*-1 polyadenylated RNA in mammary tumours varies from 2.6 to 3.8 kb. Polyadenylated RNA (5 mg) from mammary tumours with MMTV proviral integrations at the *int*-1 locus was subjected to electrophoresis in 1% agarose gels containing 2.2 M formaldehyde[13] and hybridized with probe C. Tumour numbers are indicated on top (see Fig. 3 for the positions of the proviruses within *int*-1). Tumours 24, 44 and 13 (and also tumours 17, 18, 21, 35, 36, 37, 39, 45) contain a 2.6-kb RNA hybridizing with probe C. Tumour 11 has a 3.2-kb RNA and tumours 12 and 15 have a 3.8-kb RNA species. *c*, Sandwich hybridization demonstrates covalent linkage of *int*-1 sequences to MMTV LTR sequences in tumour 12. DNA from a bacteriophage clone covering the normal *int*-1 region from positions −20 to −3.4 was digested with *Eco*RI and subjected to electrophoresis (lane *B*), next to a marker lane (A) containing wild-type λ DNA digested with *Hin*dIII. DNA was transferred to nitrocellulose filters (several identical filters were prepared from one gel). Direct annealing to radioactive probe C identified a 5.6-kb *Eco*RI fragment (arrow) that contained the *int*-1 transcriptional domain (lane *C*). (This fragment maps from the *Eco*RI site at position −9 in *int*-1 to an *Eco*RI linker at position −3.4.) Other filters were first incubated with unlabelled RNA from tumour 13 (lane *D*) or tumour 12 (lane *E*). These filters were washed and then hybridized to a radioactive probe from the MMTV LTR (a 1.4-kb *Pst*I fragment containing all but 10 bp of the LTR). Only the filter that was annealed first with the RNA from tumour 12 shows hybridization with the *Eco*RI fragment that also hybridized with probe C. Lanes *A* and *B* are photographs of the ethidium-bromide stained gel, the other lanes are autoradiograms.

Methods: 20 μg of poly(A)$^+$ RNA was resuspended in 2 ml of an annealing mix consisting of 50% formamide, 0.5 M NaCl, 20 mM PIPES *p*H 6.8, 5 mM Na_2EDTA, 0.4% SDS, 250 μg ml^{-1} poly(A), 2×Denhardt's solution, and 200 μg ml^{-1} carrier yeast RNA. This solution was applied to the filter-bound DNA and incubated for 24 h at 41 °C. The filter was washed twice at 53 °C for 15 min with 0.1×SSC, 0.1% SDS. The filter was blotted dry, annealed to MMTV–LTR probe (2×10^6 c.p.m.), washed twice for 1 h at 37 °C with 0.1×SSC, 0.1% SDS, dried and autoradiographed.

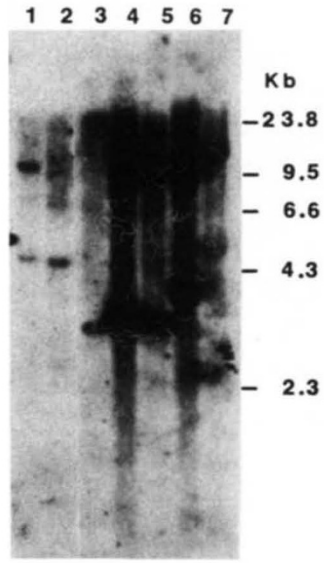

Fig. 5 Sequences homologous to *int*-1 are present in all vertebrates and *Drosophila*. Cellular DNA (10–15 μg) from the indicated sources was digested with *Bam*HI, subjected to electrophoresis in an 0.8% agarose gel, transferred to nitrocellulose filters, and annealed with probe C in 37.5% formamide, 3×SSC, at 42 °C. After washing in 2×SSC at 50 °C, the filter was exposed to X-ray film for 6 days. Lane 1, adult *Drosophila melanogaster* (Oregon-S strain); lane 2, fish (*X. helleri*); lane 3, chicken erythrocytes (strain 15×7); lane 4, mouse liver (BALB/c strain); lane 5, rat hepatoma cells (H4IIE line, ATCC); lane 6, domestic cat liver; lane 7, human mammary epithelial cells (HBL100 line; ATCC). Numbers at the right (in kb) refer to the positions of *Hin*dIII fragments of λ phage DNA present in the same gel.

DNA transformation techniques, retroviral[39–42] and cellular[43] sequences with enhancer activity have been shown to act in *cis*, over variable distances and independent of polarity, to improve the efficiency of heterologous promoters.

Our evidence that MMTV DNA can activate the expression of a previously silent gene in tumours has several of the characteristics of enhancement: the mechanism of activation operates in a manner independent of orientation, over distances of at least 10 kb, and in *cis* rather than *trans*. The basis for claiming *cis* activation is our observation that insertions between positions −8 and −6 on the *int*-1 map are associated only with transcripts of atypically large size (Fig. 4*b*), despite the persistence of the chromosome bearing a normal *int*-1 allele; we conclude that only the physically rearranged locus has been activated. Other aspects of the situation may also be germane to a consideration of mechanism. (1) The observed effect is conversion of an ostensibly silent gene to one expressed at low levels (less than 10 RNA molecules per cell), rather than to amplify expression of a gene normally transcribed. (2) Enhancer functions are likely to be cell specific[44–49] and perhaps target gene specific, so it is plausible that the MMTV enhancer works preferentially in mammary cells and upon *int*-1. (3) Initiation of MMTV transcription is greatly stimulated by glucocorticoid hormones[50], and the hormonal response displays some of the properties of enhancement[51–53]; hence it is possible that activation of *int*-1 is also affected by physiological doses of hormone. This final point should be amenable to direct test by growing mouse mammary tumour cells with *int*-1 insertions in controlled conditions in culture.

The use of an MMTV enhancer function to activate expression of *int*-1 could explain the uniform orientations of proviruses on each side of the proposed transcriptional unit (see Fig. 3). Enhancers are thought to act only or preferentially upon proximal promoters[54,55]; if the MMTV enhancer is located on the 5′ side of the initiation site for viral RNA, within the 1.2-kb U3 sequence of the LTR, then proviruses would require the orientations observed to avoid interposing an MMTV promoter between the enhancer and its *int*-1 target.

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1. Varmus, H. E. in *Mobile Genetic Elements* (ed. Shapiro, J. A.) 411–503 (Academic, New York, 1983).
2. Varmus, H. E., Quintrell, N. & Ortiz, S. *Cell* **25,** 23–36 (1981).
3. Jenkins, N. A., Copeland, M. G., Taylor, B. A. & Lee, B. K. *Nature* **293,** 370–374 (1981).
4. Copeland, N. G., Jenkins, N. A. & Lee, B. K. *Proc. natn. Acad. Sci. U.S.A.* **80,** 247–249 (1983).
5. Jaenisch, R. *et al. Cell* **32,** 209–216 (1983).
6. Kuff, E. L. *et al. Proc. natn. Acad. Sci. U.S.A.* **80,** 1992–1996 (1983).
7. Hayward, W. S., Neel, B. G. & Astrin, S. M. *Nature* **290,** 475–480 (1981).
8. Neel, B. G., Hayward, W. S., Robinson, H. L., Fang, J. & Astrin, S. M. *Cell* **23,** 323–324 (1981).
9. Payne, G. S. *et al. Cell* **23,** 311–322 (1981).
10. Payne, G. S., Bishop, J. M. & Varmus, H. E. *Nature* **295,** 209–213 (1982).
11. Noori-Daloii, M. R., Swift, R. A., Kung, H. J., Crittenden, L. B. & Witter, R. L. *Nature* **294,** 574–575 (1981).
12. Fung, Y. K. T., Fadly, A. M., Crittenden, L. B. & Kung, K. J. *Proc. natn. Acad. Sci. U.S.A.* **78,** 3418–3422 (1981).
13. Nusse, R. & Varmus, H. E. *Cell* **31,** 99–109 (1982).
14. Shank, P. R., Cohen, J. C., Varmus, H. E., Yamamoto, K. R. & Ringold, G. M. *Proc. natn. Acad. Sci. U.S.A.* **75,** 2112–2116 (1978).
15. Donehower, L. A., Huang, A. L. & Hager, G. L. *J. Virol.* **37,** 226–238 (1981).
16. Dunn, A. R. & Hassel, J. A. *Cell* **12,** 23–36 (1977).
17. Lane, M. A., Sainten, A. & Cooper, G. M. *Proc. natn. Acad. Sci. U.S.A.* **78,** 5185–5189 (1981).
18. Bishop, J. M. & Varmus, H. E. in *The Molecular Biology of Tumor Viruses. Pt III* (eds Weiss, R. A., Teich, N., Varmus, H. E. & Coffin, J. M.) 999–1109 (Cold Spring Harbor Laboratory, New York, 1982).
19 Shilo, B. Z. & Weinberg, R. A. *Proc. natn. Acad. Sci. U.S.A.* **78,** 6789–6797 (1981).
20 Rowley, J. D. *Nature* **301,** 290–291 (1982).
21 Dofuku, R., Utakoji, T. & Matsuzawa, A. *J. natn. Cancer Inst.* **63,** 651–656 (1979).
22 Cox, D. R., Sawicki, J. A., Yee, D., Appella, E. & Epstein, C. J. *Proc. natn. Acad. U.S.A.* **79,** 1930–1934 (1982).
23. Crews, S., Barth, R., Hood, L., L., Prehn, J. & Calame, K. *Science* **218,** 1319–1321 (1982).
24. Sakaguchi, A. Y., Lalley, P. A. & Naylor, S. L. *Somat. Cell Genet.* **9,** 391–406 (1983).
25. Bingham, P. M., Levis, R. & Rubin, G. M. *Cell* **25,** 693–704 (1981).
26. Fung, Y. K., Lewis, W. G., Crittenden, L. B. & Kung, H.-J. *Cell* **33,** 357–368 (1983).
27. Tsichlis, P. N., Gunter-Strauss, P. & Hu, L. F. *Nature* **302,** 445–449 (1983).
28. Peters, G., Brooke, S., Smith, R. & Dickson, C. *Cell* **33,** 369–377 (1983).
29. Cohen, J. C., Shank, P., Morris, V. L., Cardiff, R. & Varmus, H. E. *Cell* **16,** 333–345 (1978).
30. Coffin, J. M. in *The Molecular Biology of Tumor Viruses. Pt III* (eds Weiss, R. A., Teich, N., Varmus, H. E. & Coffin, J. M.) 1109–1203 (Cold Spring Harbor Laboratory, New York, 1982).
31. Sheng Ong, G. L., Keath, B. J., Piccoli, S. P. & Cole, M. D. *Cell* **31,** 443–452 (1982).
32. Banerji, J., Rusconi, S. & Schnaffer, W. *Cell* **27,** 299–308 (1981).
33. Moreau, P., Hen, R., Everett, R. & Gaub, M. P. *Nucleic Acids Res.* **9,** 6047–6069 (1981).
34. DeVilliers, J. & Schaffner, W. *Nucleic Acids Res.* **9,** 6251–6264 (1981).
35. Capecchi, M. R. *Cell* **22,** 479–488 (1980).
36. Weiher, H., Konig, M. & Gruss, P. *Science* **219,** 626–631 (1983).
37. Lusky, M., Berg, L., Weiher, H. & Botchan, M. *Molec. Cell. Biol.* **3,** 1108–1122 (1983).
38. Fromm, M. & Berg, P. *Molec. cell. Biol.* **3,** 991–999 (1983).
39. Levinson, B., Khoury, G., Vande Woude, G. & Gruss, P. *Nature* **295,** 568–572 (1982).
40. Kriegler, M. & Botchan, M. *Molec. cell. Biol.* **3,** 325–339 (1983).
41. Jolly, P. J., Esty, A. C., Sbramani, S., Friedmann, T. & Verma, I. M. *Nucleic Acids. Res.* **11,** 1855–1872 (1983).
42. Luciw, P., Bishop, J. M., Varmus, H. E. & Capecchi, M. *Cell* **33,** 705–716 (1983).
43. Conrad, S. E. & Botchan, M. *Molec. cell. Biol.* **2,** 949–965 (1982).
44. Fujimura, F. K., Deininger, P. L., Friedman, T. & Linney, E. *Cell* **23,** 809–814 (1981).
45. Katinka, M., Vasseur, M., Montreau, N., Yaniv, M. & Blangy, D. *Nature* **290,** 720–722 (1981).
46. Sekikawa, K. & Levine, A. J. *Proc. natn. Acad. Sci. U.S.A.* **78,** 1100–1104 (1981).
47. Laimins, L. A., Khoury, G., Gorman, C., Howard, B. & Gruss, P. *Proc. natn. Acad. Sci. U.S.A.* **79,** 6453–6456 (1982).
48. Gillies, S. D., Morrison, S. L., Oi, V. T. & Tonegawa, S. *Cell* **33,** 717–728 (1983).
49. Banerji, J., Olson, L. & Schaffner, W. *Cell* **33,** 729–740 (1983).
50. Varmus, H. E., Ringold, G. & Yamamoto, K. R. in *Glucocorticoid Hormone Action* (eds Baxter, J. D. & Rousseau, G. G.) 253–289 (Springer, New York, 1979).
51. Chandler, V. L., Maler, B. A. & Yamamoto, K. R. *Cell* **33,** 489–499 (1983).
52. Hynes, N., Van Ooyen, A. J. J., Kennedy, N., Herrlich, P., Ponta, H. & Groner, B. *Proc. natn. Acad. Sci. U.S.A.* **80,** 3637–3641 (1983).
53. Majors, J. E. & Varmus, H. E. *Proc. natn. Acad. Sci. U.S.A.* **80,** 5866–5870 (1983).
54. Wasylyk, B., Wasylyk, C., Augereau, P. & Chambon, P. *Cell* **32,** 503–514 (1983).
55. DeVilliers, J., Olson, L., Banerji, J. & Schaffner, W. *Cold Spring Harbor Symp. Quant. Biol.* **47,** 911–920 (1982).
56. Hynes, N. E., Kennedy, N., Rahmsdorf, V. & Groner, B. *Proc. natn. Acad. Sci. U.S.A.* **78,** 2038–2041 (1981).
57. Majors, J. E. & Varmus, H. E. *Nature* **289,** 253–258 (1981).

Correlation between segmental flexibility and effector function of antibodies

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Mouse monoclonal anti-dansyl antibodies with the same antigen-binding sites but different heavy chain constant regions were generated. The extent of segmental flexibility in times of nanoseconds and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least for IgG1 and IgE. Hence, the effector functions of immunoglobulin isotypes may be controlled in part by the freedom of movement of their Fab arms.

IMMUNOGLOBULIN G is a flexible Y-shaped molecule. The two antigen-binding Fab units of IgG are joined to an Fc unit at a hinge that allows the angle between the Fab parts to vary over a broad angular range, as shown by hydrodynamic[1], electron microscopic[2,3], and X-ray crystallographic studies[4–6]. Nanosecond fluorescence polarization measurements have demonstrated that immunoglobulin molecules exhibit segmental flexibility in the nanosecond time range[7–9]. Segmental flexibility is likely to be important in enabling immunoglobulins to bind optimally to multivalent antigens and to carry out certain effector functions. Studies of polyclonal antibody populations have suggested that immunoglobulin classes differ in their degree of segmental flexibility[10–11]. We have explored the relationship between hinge motion and effector function in specially constructed families of homogeneous immunoglobulins. Mouse monoclonal anti-dansyl antibodies with the same antigen-combining sites but different heavy chain constant regions (Fig. 1) were generated by selecting somatic variants in hybridoma cell lines. The extent of segmental flexibility in times of nanoseconds (measured by fluorescence spectroscopy) and the capacity to fix complement were greatest for IgG2b, intermediate for IgG2a, and least for IgGl and IgE. Hence, the effector functions of immunoglobulin isotypes may be controlled in part by the freedom of movement of their Fab arms.

In most antibody-producing hybridoma cell lines, variant cells arise which produce antibody containing a different heavy chain[12]. For example, cells producing IgG2b sometimes arise from a cell line producing IgGl. The frequency of these heavy-chain switch variants is ordinarily low (10^{-5}–10^{-6} per generation). These rare cells can be separated by fluorescence-activated cell sorting on the basis of the appearance of a different heavy chain on their cell surface[13,14]. In these switch variants, the light chain remains the same, whereas V_H becomes joined to a different C_H. Consequently, the antigen-combining site is the same as in the parent, despite the change in the heavy-chain constant region. Many of these switch variants are stable and can be cloned.

We chose to generate a family of homogeneous mouse anti-dansyl (DNS) antibodies for two reasons. First, the fluorescence emission spectrum of the bound dansyl chromophore is very responsive to the polarity of its environment[15], making it a convenient and sensitive indicator of whether the antigen-combining site of the switch variant antibody is in fact the same as that of the parent line. Second, the bound dansyl chromophore has an excited state lifetime suitable for determining the segmental flexibility of immunoglobulin isotypes by nanosecond fluorescence polarization spectroscopy[16]. Variant hybridoma cell lines producing different immunoglobulin isotypes were

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