The mouse homolog of the hst/k-FGF gene is adjacent to int-2 and is activated by proviral insertion in some virally induced mammary tumors

(oncogenes/fibroblast growth factors/mouse mammary tumor virus/proviral activation/breast cancer)

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ABSTRACT The fibroblast growth factor-related protooncogenes, int-2 and hst/k-FGF, are within 17 kilobase pairs of one another on mouse chromosome 7 and are in the same transcriptional orientation. Approximately 70% of tumors induced in BR6 mice by mouse mammary tumor virus have proviral insertions adjacent to the int-2 gene. We find that the murine homolog of the hst/k-FGF gene can also be transcriptionally activated by the insertion of mouse mammary tumor virus DNA either upstream or downstream of the gene. In most tumors, only one of these adjacent genes is activated, but in some cases both genes are expressed. One of the hst-expressing tumors also has a virally activated int-3 gene. At least five distinct cellular genes (int-1, -2, -3, -4, and hst/k-FGF) can therefore contribute, either singly or in concert, to the development of histologically indistinguishable mammary tumors in mice infected by mouse mammary tumor virus.

The int-2 and hst (also known as HSTF1, KS3, and k-FGF) gene products have been shown by sequence similarity to belong to a family of related proteins of which the prototype is basic fibroblast growth factor (1-4). Both genes are also considered to be protooncogenes, but for different reasons. int-2 was first identified within a common region for retroviral integration in tumors induced by mouse mammary tumor virus (MMTV), where it was subsequently shown that disruption of the locus by a provirus had activated the transcription of the gene (5, 6). In contrast, hst was identified as a dominant transforming gene when introduced into NIH 3T3 cells by DNA transfection (7, 8). In separate studies, high molecular weight DNAs extracted from a human stomach cancer (hst/HSTF1), a Kaposi sarcoma (KS3/k-FGF), and a melanomas, as well as from some normal tissues, were all shown to contain the same transforming gene, yet there was no evidence that it was expressed or played a direct role in the genesis of the original tumors (7-10). One possible explanation is that the expression of the gene is normally suppressed by a cis-acting element and that rearrangement or dissociation of these sequences during DNA preparation and transfection might permit the expression of the gene.

As well as being structurally related, the hst gene and the human homolog of int-2 have been shown to map at approximately the same position on chromosome 11, at band q13 (9, 11-13). Indeed, it now appears that these genes are within 40 kilobases (kb) of one another and are consistently coamplified in about 10-20% of human breast cancers (13-18). This close linkage of the human genes prompted us to reexamine previously isolated clones of the genomic DNA flanking the mouse int-2 locus. In particular, we had been investigating examples of MMTV-induced mammary tumors in which a provirus had integrated within the vicinity of the int-2 gene but did not appear to activate int-2 transcription. We now show that in two of these tumors the virus has activated a different cellular gene and that this gene is the murine homolog of hst. These data suggest that hst expression can directly influence the genesis of a naturally occurring tumor.

MATERIALS AND METHODS

Tumor DNA and RNA. The mouse mammary tumors discussed in this study were from the series analyzed in an earlier report (19). High molecular weight tumor DNA was prepared as described previously (5, 6). Total RNA was isolated by guanidinium thiocyanate extraction and centrifugation through cesium chloride, and the poly(A)+ fraction was enriched by chromatography on poly(U)-Sepharose (6, 19).

The analysis of DNA by restriction enzyme digestion, agarose gel electrophoresis, and blot hybridization followed standard procedures that have been described in detail elsewhere (5, 6, 19). For RNA analyses, 5-μg aliquots of poly(A)+ RNA (or 20 μg of total cellular RNA) were denatured in the presence of formaldehyde at 60°C and fractionated in agarose gels containing 2.2 M formaldehyde (19, 20). The separated RNAs were transferred to nylon membranes by blotting in 20× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate) and cross-linked by UV irradiation.

Nucleic Acid Hybridization. 32P-labeled DNA probes were prepared from isolated restriction fragments by either nick-translation or priming with random oligonucleotides (Amer sham). Hybridizations were performed in the presence of 50% (vol/vol) formamide at 42°C for DNA blots and at 50°C for RNA blots (6, 19). Filters were normally washed at high stringency at 65°C in 0.1× NaCl/Cit and 0.1% NaDodSO4, but, for cross-species hybridization, the stringency was reduced to 2× NaCl/Cit at 50°C. In some experiments, RNA blots were hybridized with an antisense RNA probe for int-2 prepared by transcribing cloned third exon sequences (HindIII–BamHI fragment) with phage SP6 polymerase (21). Other probes used in these studies were the 0.79-kb EcoRI fragment from human hst, designated probe c in ref. 10, and a 1.5-kb EcoRI–BamHI fragment from probe b for the int-3 locus (22).

EC Cells. Mouse F9 embryonal carcinoma (EC) cells were maintained as previously described and treated with retinoic acid, N6,O2'-dibutyryladenosine 3',5'-cyclic monophosphate (Bt2cAMP), and isobutylmethylxanthine to induce differentiated characteristics (21).

Abbreviations: MMTV, mouse mammary tumor virus; LTR, long terminal repeat; Bt2cAMP, N6,O2'-dibutyryladenosine 3',5'-cyclic monophosphate.

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**Recombinant DNA Methods.** Recombinant DNA clones were isolated from a λ library as described (5, 6). The library, originally constructed in Leroy Hood's laboratory (California Institute of Technology, Pasadena), contained a partial Hae III/Alu I digest of BALB/c mouse DNA inserted into the Charon 4A vector. A cosmid library (generously provided by Dimitris Kioussis, National Institute for Medical Research, London) was also screened for hst and int-2 genomic sequences. This library consisted of partial Sau3A-digested C57BL/6 T-cell DNA in the COS202 vector (23). Subclones of selected DNA restriction fragments were prepared in appropriate plasmid vectors by standard procedures (20). DNA sequence analyses on specific segments of DNA are described in detail elsewhere (24).

**RESULTS**

**Location of Proviruses in the int-2 Locus.** In a previous study, we surveyed a series of 30 spontaneous mammary tumors, arising in the BR6 strain of mouse, for proviral disruption and transcriptional activation of the int-1 and int-2 genes (19). As the limits of proviral influence have not been clearly defined, multiple combinations of restriction enzymes and genomic DNA probes were used to maximize the range of the analyses. In most cases, this established the location and orientation of the relevant MMTV proviruses, typically either 5' or 3' to the coding domains of the affected gene and at distances of up to 15 kb (refs. 19 and 25; Fig. 1). For int-2, the various enzyme/probe combinations spanned over 50 kb of mouse DNA, and this entire region is now represented in an overlapping set of λ DNA clones (ref. 25; Fig. 1).

Out of the 30 tumors analyzed in this way, 27 had sustained transcriptional activation of int-1 or int-2 and frequently both (19). Only three tumors, designated S40, E127A, and T103, scored negative for both genes. However, with one particular enzyme/probe combination (Kpn I-digested DNA with probe j; see Fig. 1), tumor S40 yielded both the expected >25-kb restriction fragment and a new fragment of ≈17.5 kb, that was consistent with insertion of MMTV proviral DNA downstream of the int-2 gene.

To confirm the location and orientation of this provirus, a more detailed restriction map was constructed for the genomic DNA contained in the corresponding λ clone, A1, and additional unique-sequence DNA probes were subcloned into plasmid vectors. The analysis of tumor S40 DNA with the probe designated EEI in Fig. 1 produced novel virus-host junction fragments with the enzymes BamHI, Sac I, and Xba I, as well as reproducing the result obtained with Kpn I and probe j (Fig. 2a). From the sizes of the novel fragments, the known restriction map of the BR6 strain of MMTV (26), and analogous data obtained using probe HHI (data not shown), it was possible to deduce that the MMTV provirus in tumor S40 was apparently complete and in the position and transcriptional orientation depicted in Fig. 1. Moreover, the intensities of the novel bands on the Southern blot, relative to those from the normal allele, suggested that the tumor was essentially clonal for integration at this locus (Fig. 2a).

**Provirus Adjacent to the Mouse hst Gene.** It was initially assumed that the MMTV provirus in tumor S40 must represent an extreme example of activation of the int-2 gene, particularly as it has been shown that a provirus at an equivalent distance upstream of the gene is capable of activating transcription (25). However, as judged by blot hybridization of poly(A)⁺ RNA (Fig. 3a), as well as more sensitive RNase protection techniques (ref. 21; data not shown), there was no detectable expression of int-2 in tumor S40. A number of possibilities were therefore considered: (i) that the location of the MMTV provirus close to int-2 was coincidental and had no bearing on tumorigenesis, (ii) that transcription of the int-2 gene had indeed been activated but was subsequently down-regulated during tumor progression (27), and (iii) that the provirus was affecting a different cellular gene.

The latter situation was the most readily testable, for example, by hybridizing tumor RNA with probes from around the S40 integration site (see below). However, we were also intrigued by the possibility that the gene in question might be the mouse homolog of hst, given the close linkage of int-2 and hst on human chromosome 11 (9, 11–13, 17, 18). A 0.79-kb genomic DNA probe encompassing the second exon of human hst (10) was found to hybridize to the A1 clone but not to any of the other λ clones from the mouse int-2 locus. More specifically, this cross-reactivity was localized to a segment of λ1 DNA contained within the 1.65-kb HindIII

![Fig. 1. Physical linkage of mouse int-2 and hst genes. The linear map of mouse genomic DNA shows the sites of cleavage for the restriction enzymes BamHI (B), Bgl II (Bg), EcoRI (E), HindIII (H), Kpn I (K), Sac I (S), and Xba I (X). The solid boxes represent coding exons for the respective genes, and the shaded boxes beneath the map depict genomic DNA restriction fragments used as hybridization probes. Each arrowhead above the map shows the position and transcriptional orientation of an integrated MMTV provirus in the numbered mammary tumor, with the filled symbols signifying the tumors in which the hst gene is expressed. The approximate extents of two of the recombinant DNA clones, A1 and COS 16, that were used in the construction of the map are indicated.](image)
**FIG. 2.** An MMTV provirus adjacent to mouse hst sequences in tumor S40. (a) Southern blot of tumor S40 DNA cut with the restriction enzymes BamHI (B), Bgl II (Bg), HindIII (H), Kpn I (K), Pst I (P), Sac I (S), and Xba I (X). The blot was hybridized with the EE1 probe depicted in Fig. 1. Novel junction fragments created by proviral insertion are indicated by arrowheads, and the numbers on the right indicate the positions and sizes of HindIII-digested DNA markers. (b) Southern blot in which DNA from the A1 clone was digested with Xba I (X), HindIII (H), or EcoRI (E) and hybridized at reduced stringency to a 0.79-kb EcoRI fragment of genomic DNA from the human hst locus (10). The 1.65-kb HindIII fragment that we have designated HH1 is indicated. (c) The nucleotide sequence is shown for a segment of the HH1 fragment that has 88% sequence similarity with the second exon of human hst (from nucleotides 3500–3620 in the genomic sequence reported in ref. 10). Intron sequences are in lowercase and the boundaries of the exon are boxed.

fragment designated HH1 (Fig. 2b). DNA sequence analysis of HH1 identified a segment that is identical at 91 of the 104 residues that comprise the second exon of human hst (Fig. 2c). We have subsequently determined the exact location and organization of other exons of the mouse hst gene, as depicted in Fig. 1, by determining the complete genomic DNA sequence (24).

**Expression of the hst Gene in Mouse Mammary Tumors.** Having established that the HH1 fragment from A1 contained part of the mouse hst gene, we tested whether it would hybridize to specific RNAs expressed in tumor S40. As shown in Fig. 3b, this probe detected a major transcript of ~3.2 kb in S40 but not in two other tumors included in the analysis (Fig. 3b). As a further control for these experiments, we used RNA from the F9 EC cell line, since these cells are known to express int-2-encoded RNA when induced with retinoic acid and Bt2cAMP (ref. 21; and Fig. 3a). With hst, however, the 3.2-kb transcript was expressed in the undifferentiated F9 cells, and its level declined when the cells were treated with retinoic acid (Fig. 3b). A similar result has recently been reported by Yoshida et al. (28).

It was also important to establish whether the expression of hst in tumor S40 was an isolated case or whether other tumors in the original series expressed the gene. As exemplified in Fig. 4, we surveyed a total of 22 tumors, including many that had been shown to express both int-1 and int-2, as well as the two examples (E127A and T103) that do not express either gene (19). Three additional hst-positive cases were identified, namely W106 and E127A (Fig. 4) and Z79 (data not shown), all of which contained substantially more hst-encoded RNA than was apparent in S40. This left only one tumor out of the original series (i.e., T103) that had not yielded evidence for gene activation by MMTV. However, when the RNA blot used in Fig. 4 was rehybridized with a riboprobe for int-2, a faint signal was detected in tumor T103 after prolonged exposure of the autoradiograph. The interpretation of this result remains unclear since the single band observed does not conform with the complex pattern of transcripts normally associated with int-2. It is nevertheless...
To date, the LTR, in the W106, suggesting restriction the tumors however, mediated activation of not therefore reexamined in of both showed 10 kb int-2 mammary mammary cells that had induced BR6 after 10 kb int-3. FIG. 4. In attempts quasi-clonal tumors, not shown). Thus, it has since been extended discontinuities a of hst. The int-2 cloned in the BR6 tumors, all but three conformed to the pattern associated with enhancement of gene expression by the viral LTRs. Thus, proviruses located 5' to the gene are in the opposite transcriptional orientation to int-2, whereas proviruses located 3' of the coding domain are transcribed in the same direction as the gene (6, 19). The exceptions include cases where the provirus is in a ‘‘promoter insertion’’ mode, driving int-2 expression. MMTV promoter (19, 29), and an unusual situation where a deleted provirus has been mapped downstream of the gene but in the opposite transcriptional orientation (D. Baines, R. Moore, G.P., S.B., R.S., and C.D., unpublished results). This tumor (U153) expresses additional int-2-encoded RNAs that terminate within the viral LTR but apparently initiate at the normal int-2 promoters (Fig. 3a). Although it defies the accepted norms for activation of int-2, because the viral promoter in the 3' LTR comes between the enhancer and the target gene, this deleted provirus would be in the appropriate orientation to enhance expression of hst, albeit at a distance of 12 kb. However, as indicated in Fig. 3b, there was no detectable expression of hst RNA in tumor U153. Similarly, the provirus in tumor S40, and indeed the solo LTR in E127A, would be in the appropriate orientation for enhancing int-2 expression, but they do not appear to do so. Such observations, coupled with the noticeable lack of proviral insertions within the 10 kb separating the integration sites in U153 and S40, are intriguing. If the mouse hst gene is under the control of a cis-acting suppressor, as suggested for human hst, then it is conceivable that the lack of insertions in this region may reflect an unusual chromatin conformation associated with the negative element. The proviral activation in tumor S40 could therefore represent a dissociation of the gene from its silencer rather than classical promoter insertion, since the hst transcript observed in the tumor is superficially similar to the presumably normal RNA expressed in F9 cells. Such an interpretation might also explain the low levels of hst RNA detected in tumor S40 compared to those seen in E127A. In the latter

**FIG. 4.** Expression of hst, int-2, and int-3 in a series of MMTV-induced mammary tumors. Poly(A)+ RNAs (5 μg) from the indicated BR6 mouse mammary tumors were analyzed with probes for hst, int-2, and int-3. Samples (20 μg) of total RNA from F9 cells before (F9) and after induction with retinoic acid and Bt2-cAMP (F9 D+) were included as controls. The sizes of the major transcripts are indicated to the right. Note that the smaller 1.8- and 1.6-kb species of int-2 RNA are not included.

**FIG. 5.** Disruption of hst DNA in tumor E127A. A Southern blot is shown of tumor E127A DNA digested with the enzymes BamHI, (B), Bgl II (Bg), EcoRI (E), HindIII (H), Pat I (P), or Xba I (X) and hybridized with probe HH3 (see Fig. 1). Additional bands created by insertion of MMTV LTR sequences are indicated by arrowheads.

**DISCUSSION**

To date, four presumed protooncogenes (int-1, -2, -3, and -4) have been identified on the basis of their proximity to and activation by integrated MMTV DNA in mouse mammary tumors (30). Despite the similar strategies in their identification, these genes are quite distinct; they reside on different mouse chromosomes and encode unrelated proteins (30–32). We have now found a fifth gene that can be activated by MMTV; it is both related to and adjacent to int-2 on mouse chromosome 7. Since there are at least two examples of proviral integration in the flanking chromosomal DNA, there is an argument for designating the locus as int-5. However, since the nomenclature is already confusing, we have elected to retain the acronym hst in this report, although it may be considered inappropriate for the mouse homolog.

Previous studies have suggested that proviral activation of int-2 leads to expression of the normal gene product, in that no examples have been observed in which the protein coding domain of the gene has been perturbed (ref. 19 and unpublished results). Moreover, of the proviruses mapped within int-2 in the BR6 tumors, all but three conformed to the pattern associated with enhancement of gene expression by the viral LTRs. Thus, proviruses located 5' to the gene are in the opposite transcriptional orientation to int-2, whereas proviruses located 3' of the coding domain are transcribed in the same direction as the gene (6, 19). The exceptions include cases where the provirus is in a ‘‘promoter insertion’’ mode, driving int-2 expression. MMTV promoter (19, 29), and an unusual situation where a deleted provirus has been mapped downstream of the gene but in the opposite transcriptional orientation (D. Baines, R. Moore, G.P., S.B., R.S., and C.D., unpublished results). This tumor (U153) expresses additional int-2-encoded RNAs that terminate within the viral LTR but apparently initiate at the normal int-2 promoters (Fig. 3a). Although it defies the accepted norms for activation of int-2, because the viral promoter in the 3' LTR comes between the enhancer and the target gene, this deleted provirus would be in the appropriate orientation to enhance expression of hst, albeit at a distance of 12 kb. However, as indicated in Fig. 3b, there was no detectable expression of hst RNA in tumor U153. Similarly, the provirus in tumor S40, and indeed the solo LTR in E127A, would be in the appropriate orientation for enhancing int-2 expression, but they do not appear to do so. Such observations, coupled with the noticeable lack of proviral insertions within the 10 kb separating the integration sites in U153 and S40, are intriguing. If the mouse hst gene is under the control of a cis-acting suppressor, as suggested for human hst, then it is conceivable that the lack of insertions in this region may reflect an unusual chromatin conformation associated with the negative element. The proviral activation in tumor S40 could therefore represent a dissociation of the gene from its silencer rather than classical promoter insertion, since the hst transcript observed in the tumor is superficially similar to the presumably normal RNA expressed in F9 cells. Such an interpretation might also explain the low levels of hst RNA detected in tumor S40 compared to those seen in E127A. In the latter
tumor, the expression of hst is presumably enhanced by the downstream LTR.

However, the fact that hst is also expressed in tumors Z79 and W106, in the apparent absence of any disruption within the present limits of the hst locus, challenges this interpretation. Unless these tumors contain as yet undetected proviruses downstream of hst, we must assume that hst is being activated at a distance by the previously mapped proviruses in the 3’ untranslated region of int-2. This presents a paradox since other int-2-positive tumors, in which proviruses have been located very close to those in Z79 and W106, do not express detectable levels of hst RNA (Fig. 4, tumors D124 and E146). A more comprehensive survey of additional mammary tumors may help to resolve this issue.

Further studies might also indicate whether any tumors have sustained proviral disruptions in both int-2 and hst, analogous to the previously reported activation of both int-1 and int-2 in ostensibly clonal populations of cells (19), and the dual activation of hst and int-3 seen here in tumor E127A. Disruption of two genes can be explained if each event provided a selective advantage to the developing tumor cell. Although tumors Z79 and W106 featured among the examples that expressed both int-1 and int-2 (19), it is not clear whether coexpression of hst in these tumors was coincidental or provided an additional stimulus to neoplastic growth. This becomes an important question given the structural and perhaps functional similarities between hst and int-2 and the possibility that mammary epithelial cells might respond to both factors, through the same or separate receptors. Interactions between these presumed growth factors and cognate receptors could also prove significant for the growth and differentiation of EC cells and other embryonic lineages. Given the strict spatial and temporal regulation of int-2 expression during mouse development and the suggestion of multiple inductive roles, it will be interesting to determine the patterns of hst expression (33, 34). Whatever the answers, the independent activation of hst in tumors S40 and E127A is one of the strongest pieces of evidence to implicate hst in a naturally occurring cancer and suggests that at least five distinct cellular genes (int-1, -2, -3, -4, and hst), either singly or in various combinations, can contribute to the genesis of pathologically indistinguishable mammary tumors.

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