Fgf-8, Activated by Proviral Insertion, Cooperates with the *Wnt-1* Transgene in Murine Mammary Tumorigenesis

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We have used mouse mammary tumor virus (MMTV) infection of *Wnt-1* transgenic mice to accelerate mammary tumorigenesis and to molecularly tag insertionally activated proto-oncogenes that cooperate oncogenically with *Wnt-1* (G. M. Shackleford, C. A. MacArthur, H. C. Kwan, and H. E. Varmus, Proc. Natl. Acad. Sci. USA 90:740–744, 1993). Here we report the identification and characterization of a 31-kb genomic locus that contains clonal MMTV integrations in 8 of 80 mammary tumors from MMTV-infected *Wnt-1* transgenic mice. Two genes were identified within this locus, one of which was transcriptionally activated by MMTV insertions. This activated gene is identical to androgen-induced growth factor (AIGF/*Fgf-8*) (A. Tanaka, K. Miyamoto, N. Minamino, M. Takeda, B. Sato, H. Matsuo, and K. Matsumoto, Proc. Natl. Acad. Sci. USA 89:8928–8932, 1992), the eighth member of the fibroblast growth factor (FGF) family. Transcriptional activation of *Fgf-8* was found in all tumors with MMTV insertions in this locus. *Fgf-8* mRNA was absent in normal mammary glands and was detected only in adult testis and ovary and in midgestational embryos. The sequences of *Fgf-8* genomic and cDNA clones revealed five coding exons, in contrast to the three coding exons found in other FGF genes. cDNAs encoding three isoforms of the FGF-8 protein were isolated. The three corresponding mRNAs resulted from the alternative use of two 5' splice sites and two 3' splice sites for the second and third exons, respectively. These results implicate *Fgf-8* as the third FGF gene found to cooperate with *Wnt-1* in MMTV-induced murine mammary tumorigenesis, suggesting that FGFs and Wnts are strong collaborators in this process.

Ectopic expression of the *Wnt-1* proto-oncogene in the mammary glands of transgenic mice causes a diffuse mammary gland hyperplasia, followed by the stochastic development of adenocarcinomas in both males and females after a minimum latency of several months (44). These findings indicate that activation of *Wnt-1* is but one step in the multistep process of mammary tumorigenesis. Early evidence that fibroblast growth factors (FGFs) may be involved in cooperating steps comes from the finding that mouse mammary tumor virus (MMTV) insertionally activates both *Wnt-1* and *Fgf-3* in some tumors of infected nontransgenic mice (35). The cooperation of *Wnt-1* and *Fgf-3* was confirmed with the generation of bitransgenic *Wnt-1/Fgf-3* mice, which develop tumors earlier than either of the monotransgenic mice (26).

We have studied multistep murine mammary tumorigenesis by infecting *Wnt-1* transgenic mice with MMTV, expecting that infection would hasten tumor formation in these mice by insertional activation of proto-oncogenes that oncogenically cooperate with *Wnt-1* (37). As a result, activated proto-oncogenes are “tagged” by the nearby provirus, allowing their identification or isolation. Previous studies using murine leukemia virus infection of *Eμ-myc* transgenic mice have uncovered at least five distinct genetic loci that appear to collaborate with *c-myc* in lymphomagenesis (20, 45). MMTV infection of *Wnt-1* transgenic mice results in accelerated mammary tumorigenesis and increased numbers of tumors per animal (37).

Approximately 45% of the resulting tumors with clonal, tumor-specific MMTV integrations contain insertionally activated *Fgf-3* or *Fgf-4*, suggesting that the *Wnt-1* gene product can cooperate with at least two members of the FGF family (37).

Despite being able to detect clonal, tumor-specific MMTV proviruses in the remaining 55% of the tumors, we did not detect insertional activation of any previously identified targets for MMTV insertion mutations (*Fgf-3*, *Fgf-4*, *int-3*, or *Wnt-3*) in this group (37). To determine the identity of proto-oncogenes that may be activated in these tumors, we have analyzed the DNAs surrounding proviral integration sites. We report here on the cloning of a new common integration locus for MMTV that contains insertions in 10% of our mammary tumors that have clonal, tumor-specific MMTV proviruses. Of the two genes located in this region, only one is activated by MMTV insertion mutations. This activated gene was found to encode androgen-induced growth factor (42), the eighth member of the FGF family. Thus, the selective activation in this system of a third FGF gene by MMTV proviral insertion suggests that FGFs and Wnts are potent collaborators in murine mammary tumorigenesis.

MATERIALS AND METHODS

Tumor samples. All mammary tumors were derived from a previous study (37) in which we infected female *Wnt-1* transgenic mice (44) at 3 to 4 weeks of age with MMTV produced from EH-2 cells (39).

Preparation of nucleic acids. Tumor DNAs were isolated as described before (39) except that serum separation tubes were used in the extractions (43). The majority of tumor RNAs were isolated by the urea-lithium chloride method (3) as described before (38). The normal tissue RNAs and some tumor RNAs were isolated by the guanidinium isothiocyanate-acid-phenol method (9). Polyadenylated [poly(A)⁺] RNAs were purified by oligo(dT) chromatography (4). RNAs were stored as isopropanol precipitates at –20 or –80°C.

Southern and Northern (RNA) blot analyses. DNAs (10 μg) were digested with restriction endonucleases, electrophoresed, and capillary blotted to nylon membranes (Amersham Hybond-N) as previously described (39). The blots were

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UV cross-linked (11), prehybridized for 1 to 2 h at 65°C, and hybridized overnight at 65°C with ³²P-labeled DNA probes. The hybridization buffer contained 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% bovine serum albumin (fraction V), 7% sodium dodecyl sulfate (SDS), and 15% (vol/vol) formamide. Blots were washed in 40 mM sodium phosphate (pH 7.2)–1% SDS–1 mM EDTA at 65°C. Blots were exposed to Kodak XAR-5 film with intensifying screens at –80°C.

For Northern blots, RNAs were resuspended in a solution containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.0), 10 mM sodium acetate, 1 mM EDTA, 0.25 µg of ethidium bromide per ml, 0.66 M formaldehyde, and 50% (vol/vol) formamide, denatured at 65°C for 5 min, and electrophoresed in 0.8% agarose gels with the same running buffer minus ethidium bromide and formamide at 30 to 45 V for 6 to 14 h (38). Following photography, the gel was capillary blotted overnight to nylon. Cross-linking, prehybridization, hybridization, washing, and exposure were the same as for DNA except that the hybridization buffer contained 30% (vol/vol) formamide for Northern blots. Blots were stripped of probes by incubation in boiling 0.1% SDS for 3 to 5 min and rehybridized as above.

Preparation and screening of genomic libraries. Following identification of a proviral-cellular junction fragment by Southern blotting, the fragment was size-selected by agarose electrophoresis and purified by using either glass beads (Gene Clean II; Bio 101) or agarose digestion (β-Agarase; New England Biolabs) and isopropanol precipitation. The junction fragments were ligated into appropriate lambda phage vectors, depending on the size of the insert (λDash II for inserts of 10 to 20 kb, and λZap II for inserts of less than 10 kb [both from Stratagene]). Phage were prepared by using packaging extracts (Gigapack II Plus; Stratagene) and then titrated and screened on appropriate *Escherichia coli* hosts (XL-1 Blue for λZap libraries and P2392 for λDash libraries). Plaque lifts were performed as described before (36), UV cross-linked, hybridized, washed, and exposed as above for Southern blots. Putative positive clones were subjected to two to three rounds of screening to isolate clones. The inserts were obtained in Bluescript (Stratagene) plasmids, either by subcloning or by *in vivo* excision of λZap clones.

Growth and transfection of Cos-7 cells. A 6-kb *SsrII* fragment containing most of *Fgf-8* (see Fig. 5A) was subcloned into the “exon trap” vector pML53In (gift of M. Reith) (2), in both orientations. The resulting plasmids were transiently expressed in Cos-7 cells (American Type Culture Collection) by lipofectin-mediated transfection (BRL-Gibco). Cos-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (Sigma), penicillin, and streptomycin. When the cells reached 70% confluence, the medium was changed to DMEM without serum, and the DNA-lipofectin mixture was added according to the manufacturer’s directions (BRL-Gibco). After 12 h of incubation of the lipofectin-DNA with the cells, serum was added back, and the cells were grown in DMEM with 10% fetal calf serum for an additional 48 h. The cells were harvested, and total cellular RNA was prepared (9). The RNA was used to generate an *Fgf-8* cDNA, using an exon trap protocol (2).

Generation of cDNAs by RT-PCR. Reverse transcription (RT) of total cellular RNA from transiently transfected Cos-7 cells was done in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 µg of random hexamer primers per ml, and avian myeloblastosis virus reverse transcriptase (Stratagene) by incubating at room temperature for 10 min, followed by 37°C for 20 min, and then 42°C for 90 min. The resulting cDNA was amplified by nested PCR with Amplitaq (Perkin-Elmer Cetus), with an initial forward primer (5′-AAGC TCTCTACCTGGTGTGG-3′) in the 5′ insulin exon and an initial reverse primer (5′-CAGTGCCAAGGTCTGAAGGTC-3′) in the 3′ insulin exon, followed by a nested forward primer in the 5′ insulin exon (5′-GCGAAGTGGAG GATCCACAAG-3′) and a nested reverse primer in the 3′ insulin exon (5′-ACCCGGATCCAGTTGTGCCA-3′). Initial PCR conditions were 50 mM KCl–1.5 mM MgCl₂ and 40 cycles of denaturing at 94°C for 1 min, annealing at 59°C for 1 min, and extending at 72°C for 1 min. Nested PCR conditions were the same except that the annealing temperature was 50°C. The resulting *Fgf-8* cDNA product, obtained only from the plasmid with *Fgf-8* in the correct orientation, was isolated from an agarose gel, cloned into a pBluescript “T-vector” (27), sequenced, and found to contain exons 2A, 3A, 4, and 5 up to the *SsrII* site. The portion of the coding region downstream of the *SsrII* site in exon 5 was added by ligating a 160-bp *SsrII*-*SsrI* fragment.

We obtained cDNAs containing all of the known *Fgf-8* coding region (42) in two subsequent steps. First, upstream cDNAs (exons 1, 2A, and 3A; exons 1, 2A, and 3B; and exons 1, 2B, and 3A) were obtained by nested RT-PCR from murine testis RNA. The RNA was reverse transcribed as above. The first PCR was carried out with a forward primer in *Fgf-8* exon 1 (5′-CGCACCCGACCTC CCTC-3′) and a reverse primer in *Fgf-8* exon 3 (5′-GAGCTGATCCGTCAC CAGGCT-3′). A nested *Fgf-8* exon 1 forward primer (5′-CGCACCTTCGGCT TGTCC-3′) was used with the reverse *Fgf-8* exon 3 primer above or with a reverse primer that spans exons 2B and 3A (5′-CTGCTCCCTCACATGT CGCTG-3′) in the nested PCR. PCR conditions with recombinant *Pfu* DNA polymerase (Stratagene) for both the initial and nested steps were 10 mM KCl–2 mM MgCl₂ and 40 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 1 min, and extending at 72°C for 1 min. Next, these 5′ cDNA fragments (189 bp for exons 1, 2A, and 3A; 222 bp for exons 1, 2A, and 3B; and 360 bp for exons 1, 2B, and 3A) were annealed with the exon trap fragment and amplified by *Pfu* DNA polymerase with the forward primer (5′-CGCACCTTCGGCTTGTCC-3′)

in exon 1 and a reverse primer in exon 5 (5′-CGACTCCCGCTGGATTCTCT-3′) to give cDNAs containing the entire coding sequence of the three isoforms of *Fgf-8*. The conditions for this step were 10 mM KCl–2 mM MgCl₂ and 25 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extending at 72°C for 1 min. These cDNAs were cloned into pBluescript T-vectors (27). Sequencing of two of these fragments showed that they correspond to the previously described clones pSC17 (exons 1, 2A, 3B, 4, and 5) and pSC15 (exons 1, 2B, 3A, 4, and 5) (42). The third fragment is a novel isoform derived from exons 1, 2A, 3A, 4, and 5 (see Results).

Probes and sequencing. The DNA probes used for hybridization were isolated from plasmids by digestion with restriction endonucleases, agarose gel electrophoresis, and glass bead binding. Potential probes were tested for repetitive elements by blotting to nylon and hybridizing with ³²P-labeled mouse genomic DNA (41). Unique probes were used on Southern and Northern blots. In addition to the probes generated from the genomic clones and from PCR products described above, the following probes were used on blots: a 1.9-kb *PstI*-*XhoI* fragment (MMTV *gag* probe) and a 1.2-kb *BamHI* fragment (MMTV *env* probe) (39); a 475-bp murine acidic FGF/*Fgf-1* cDNA, a 475-bp murine basic FGF/*Fgf-2* cDNA, a full-length 800-bp murine *Fgf-5* cDNA, and a 600-bp murine *Fgf-6* cDNA (21); a 560-bp murine *Fgf-6* cDNA and a 1.6-kb *XbaI*-*XhoI* murine 5′ genomic *Fgf-6* fragment (14); a 693-bp rat KGF/*Fgf-7* cDNA (48); a 0.85-kb murine *Fgf-9* cDNA (gift of D. Ornitz); and a 1.3-kb *PstI* fragment containing rat glyceraldehyde-3-phosphate dehydrogenase cDNA (18).

Double-stranded plasmids containing inserts were sequenced with Sequenase version 2.0 (US Biochemicals). Both strands were sequenced with a combination of deletion subclones and *Fgf-8*-specific primers.

Nucleotide sequence accession number. The GenBank accession number of the cDNA encoding FGF-8a is U18673.

RESULTS

Detection and cloning of a new tumor-specific common insertion site for MMTV. In previous work, we showed that MMTV infection of *Wnt-1* transgenic mice accelerates mammary tumorigenesis by insertional activation of *Fgf-3* and *Fgf-4* (37). However, approximately half of the tumors that we examined did not exhibit activation of any of the proto-oncogenes known to be affected by MMTV in tumors of normal mice (including *int-3* and *Wnt-3*), despite the presence of clonal proviral insertions in these tumors (37). We reasoned that these tumors were likely to harbor unpredicted or novel proto-oncogenes activated by proviral insertion. To isolate such genes, we first sought to clone the proviral-cellular junction fragments from tumors that contained only one or two clonally integrated proviruses. Southern blot analysis of tumor DNAs with an MMTV *gag* probe identified several candidate junction fragments (Fig. 1 and data not shown).

The *XhoI*-cleaved junction fragments from tumors 95 and 111 (Fig. 1) were cloned into lambda vectors and isolated by screening with an MMTV *gag* probe. Unique cellular DNAs flanking the MMTV proviruses in these clones were isolated and used as probes on Southern blots of DNAs from 80 tumors with clonal insertions to determine if other tumors had rearrangements due to proviral insertions in these loci. Data from these experiments suggested that the insertions in tumors 95 and 111 were actually in the same locus and, furthermore, that six other tumors also contained proviruses in this region (Fig. 2 and 3 and data not shown). Hybridization of these blots with MMTV probes confirmed that the rearrangements of this locus in affected tumors were due to proviral integrations (Fig. 2 and 3 and data not shown). The apparent presence of two insertions in tumor 135 is discussed below.

Detection of expressed genes in the common insertion region. In a search for active genes in this locus, we made probes from the cloned cellular DNAs described above as well as from adjacent regions cloned subsequently for use in Northern blot analyses of tumor RNAs. Two genes were identified. A genomic clone that detected RNA from one of these genes was sequenced and found to be identical with the cDNA of androgen-induced growth factor (42), the eighth member of the FGF family. We will hereafter refer to this gene as *Fgf-8*. The second

FIG. 1. MMTV proviruses in tumors of *Wnt-1* transgenic mice. Genomic DNAs were isolated from normal mammary gland (lane C) or from mammary tumors (designated by tumor number) of MMTV-infected *Wnt-1* transgenic mice. DNAs were digested with *Xho*I, separated by agarose gel electrophoresis, capillary blotted to nylon membranes, and hybridized with a 32 P-labeled MMTV *gag* probe. All DNAs have large *gag* fragments, indicative of endogenous retroviruses in these animals. Most of the tumor samples have an additional fragment(s), indicating the presence of clonal, tumor-specific MMTV proviruses. The positions of DNA size markers (in kilobases) are indicated at the right. A diagram showing the origin of the *gag* probe used and the 5' proviral-cellular junction fragment it detects is shown below the gel. MMTV DNA is represented with bold lines. Open boxes, MMTV LTRs. Xh, *Xho*I.

gene in this locus, which we have designated *Nub-1*, is novel. The deduced *Nub-1* product is unrelated to any oncoproteins, but it has sequence similarities to two nucleolar chaparone proteins, nucleoplasmin and B23/nucleophosmin (6, 16). A description of *Nub-1*, beyond the RNA data below, will be presented elsewhere.

When small amounts of total cellular RNAs available from tumors were examined by Northern blotting for expression of these genes, we found evidence for transcriptional activation of *Fgf-8* in all tumors with insertions in this locus except tumor 104; *Fgf-8* RNA was undetectable in tumors without insertions and in normal mammary gland (Fig. 4 and data not shown). RT-PCR experiments confirmed the presence of *Fgf-8* RNA in tumor 104 and its absence in normal mammary gland (data not shown). In contrast, the RNA levels of the constitutively active *Nub-1* gene do not appear to be significantly affected by the surrounding MMTV insertions (Fig. 4). Thus, the activated expression of *Fgf-8* in multiple tumors by clonal proviral insertions suggests that this gene is causally related to tumor formation.

***Fgf-8* exon structure and alternative splicing.** To determine the physical relationship of the proviral insertions to the *Fgf-8* gene, we established its exon structure by sequencing portions of our genomic clones and comparing these with the *Fgf-8* cDNA sequences reported earlier (42). This revealed that *Fgf-8*, as represented by these cDNAs, is approximately 6.5 kb in length and has at least five coding exons (Fig. 5A), unlike other analyzed FGF genes, which have only three coding exons (1, 14, 19, 24, 28, 49, 50). All of the insertions detected were mapped upstream of *Fgf-8*, and all, except those in tumors 61 and 86, were in the opposite transcriptional orientation to

FIG. 2. MMTV proviral insertions into a new common integration locus. (A) Southern blots of DNAs from kidney as a control (lanes C) and from mammary tumors 86, 104, 111, and 135. Rearrangements of this locus in tumors were detected following digestion of the DNAs with either *Bgl*II or *Xba*I (which cut within MMTV) and hybridization of the resulting blots with the cellular probe PH 0.8 (see Fig. 3). Arrowheads identify fragments that anneal to both cellular (left panels) and MMTV *gag* (right panels) probes, demonstrating the location and orientation of the inserted provirus. (B) Southern blots of DNAs from normal mammary gland as a control (lanes C) and from mammary tumors 61, 63, 94, and 95. Rearrangements of this locus in tumors were detected following digestion of the DNAs with *Xho*I and hybridization of the resulting blots with the cellular probe XP1200 (see Fig. 3). The orientation of the MMTV provirus was determined as for panel A but with XP1200, *gag*, and *env* probes.

Fgf-8 (Fig. 3 and 5A and data not shown). Two of the insertions (tumors 63 and 95) disrupted the *Nub-1* gene, which is located approximately 5 kb upstream of *Fgf-8* in the same orientation, and two insertions (tumors 61 and 94) were upstream of both genes (Fig. 3 and data not shown).

The comparison of *Fgf-8* genomic and cDNA sequences also

FIG. 3. Map of proviral insertions in the new common integration locus. This locus was mapped with restriction digests of lambda phage clones and plasmid subclones containing inserts from this locus, and by Southern blotting of genomic DNAs with probes from the locus. The lambda phage clones are indicated at the bottom of the figure. MMTV proviral insertions were mapped by Southern blotting with probes from this locus (XP1200, PH 0.8, and PP 1.3) and from MMTV, as described in the legend to Fig. 2. In some cases, the MMTV proviral insertion site was sequenced following amplification by PCR. Arrowheads denote the locations and transcriptional orientations of the MMTV proviruses. B, *Bam*HI; Bg, *Bgl*II; RI, *Eco*RI; S2, *Sst*II; X, *Xba*I; Xh, *Xho*I.

showed the existence of at least two 5' splice sites in exon 2 and two 3' splice sites in exon 3. Alternative splicing between these sites could potentially produce RNAs encoding four isoforms of the protein (Fig. 5A to C). In exon trap experiments with an *Sst*II genomic fragment containing most of *Fgf-8* (see Materials

and Methods) and in nested RT-PCR experiments with RNA from adult murine testis and from tumor 61, we amplified and cloned cDNAs of alternatively spliced mRNAs encoding three different isoforms: FGF-8a, FGF-8b, and FGF-8c (Fig. 5A to C). We did not obtain a cDNA containing both exons 2B and 3B, which would encode the fourth potential isoform (data not shown). All three cloned versions have the same predicted signal peptide sequence and code for the same protein downstream of the alternative splice, but differ in the amino-terminal regions of the putative secreted products (Fig. 5C).

Expression of *Fgf-8* in murine tissues. We performed Northern blots of poly(A)⁺ RNA from normal murine tissues to determine the normal expression patterns of *Fgf-8* (Fig. 6). Weak expression was detected in ovary and testis, but we did not detect *Fgf-8* RNA in normal mammary gland or in any of 10 other tissues (Fig. 6). These data suggest that MMTV insertions in tumors activate this gene from a transcriptionally silent state.

Since FGFs are frequently expressed during embryogenesis (5), we looked for expression of *Fgf-8* during murine development by Northern blot analysis of total cellular RNA from whole embryos staged by gestational age. We found high expression of *Fgf-8* at 10.5 days postconception, the earliest time tested, which decreased to nearly undetectable levels by 14.5 days (Fig. 6). We did not observe *Fgf-8* expression at 16.5 or 17.5 days postconception (data not shown).

DISCUSSION

We previously showed that infection of *Wnt-1* transgenic mice with MMTV accelerates mammary tumorigenesis by insertional activation of *Fgf-3* and/or *Fgf-4*, indicating that these two members of the FGF family can cooperate with *Wnt-1* in murine mammary tumorigenesis (37). By cloning and analyzing proviral insertion sites from several tumors that lack these activations, we show here that 8 of 80 tumors with clonal, tumor-specific MMTV proviruses had MMTV insertions in a genomic region that contains *Fgf-8* (Fig. 2 and 3). Elevated levels of *Fgf-8* RNA were detected in affected tumors but not in other tumors or in normal mammary gland (Fig. 4). Analysis of the normal gene and its expression showed that *Fgf-8* contains at least five coding exons and can encode at least three different protein isoforms by alternative splicing and that RNA was present in the testes and ovaries of adults and in midgestational embryos. Together, these findings suggest that the overexpression of *Fgf-8* which accompanies MMTV insertion into this locus contributes to the formation of these tumors.

Proviral insertions. Studies of MMTV insertion sites in mammary tumors indicate that MMTV can transcriptionally activate an adjacent proto-oncogene from upstream or downstream locations in either transcriptional orientation relative to the gene, although the vast majority are classical "enhancer insertions," in which the 5' long terminal repeat (LTR) of the provirus is situated closest to the activated gene (12, 30, 33). All of the insertions described here are located upstream of *Fgf-8*; two of these (in tumors 61 and 86) were in the same orientation as *Fgf-8* (and *Nub-1*). The location of the provirus in tumor 61 might predict a large fusion transcript with *Nub-1*, starting from an MMTV LTR, but no aberrant *Nub-1* (or *Fgf-8*) RNAs were observed in this tumor. Thus, it appears that the *Fgf-8* gene is activated by an enhancer insertion mechanism in tumor 61, despite the "promoter insertion" orientation of the provirus. The identities of the additional sequences in the *Fgf-8* transcripts larger than 1.9 kb observed in several tumors (e.g., tumors 63, 86, and 135 in Fig. 4) are unknown.

Tumors 61 and 94 are unusual in that a second gene, *Nub-1*,

FIG. 4. Transcriptional activation of *Fgf-8* by MMTV insertion in tumors from infected *Wnt-1* transgenic mice. Northern blot of total cellular RNA (5 µg) from three normal tissues (adrenal gland, kidney, and mammary gland), two tumors without MMTV proviruses near *Fgf-8* (tumors 14 and 42), and eight tumors with MMTV insertions near *Fgf-8*. The blot was initially probed with a full-length *Fgf-8b* cDNA (upper panel), then with a *Nub-1* cDNA (middle panel), and finally with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (lower panel). The locations of RNA size markers are shown at right.

is found between the proviral insertions and the activated *Fgf-8* gene. The basal activity of *Nub-1* does not seem to be significantly affected by the insertions, while *Fgf-8* is activated from an apparently quiescent state. Although proviral enhancers are known to activate genes from significant distances, to our knowledge, MMTV activation of the distal, but not the proximal, gene of a linked pair has not previously been observed. The apparent selective mechanism involved is unclear but may be explained in part by the relatively high basal level of *Nub-1* expression, by possible toxicity of higher levels of Nub-1 protein, or by a discriminating compatibility of MMTV enhancers with the *Fgf-8* promoter.

Tumor 135 appears to contain two separate MMTV proviral insertions at similar locations in this locus, but not both insertions on a single chromosome (Fig. 2B). It seems unlikely that a selective advantage for tumor cell growth would result from both copies of the gene being activated by proviral insertion, since, for example, we have never seen insertional activation of the endogenous *Wnt-1* gene in MMTV-infected *Wnt-1* trans-

genic mice (37). Although a requirement for a threshold level of *Fgf-8* expression might explain the two insertions, alternative interpretations are that tumor 135 is oligoclonal, with tumor cells possessing one or the other insertion, or that a single insertion exists with a rearranged form present in some tumor cells. In addition, tumor 135 has an insertion in the *Fgf-3/Fgf-4* locus (37). This finding reintroduces the notion of possible cooperation among FGF family members (37), but oligoclonality could also explain the data.

Gene activation by MMTV insertions. Almost all tumors with insertions in this locus displayed readily discernable activation of *Fgf-8* with small amounts of total RNA by Northern analysis. Only tumor 104 required RT-PCR for detection. The cause for the wide variation in MMTV-activated *Fgf-8* gene expression is unknown, but such variation is common for genes activated by MMTV insertions (15, 31, 34, 37). Because of the lack of obvious changes in *Nub-1* RNA levels in affected tumors and the interruption of its coding domain in two tumors, alterations in *Nub-1* expression by proviral insertions do not appear to contribute to tumorigenesis. The relationship of *Nub-1* to the nucleolar proteins nucleoplasmin and B23/nucleophosmin, two abundant and well-studied molecular chaperones with no known oncogenic potential (6, 16), also does not immediately point to a role for *Nub-1* in tumorigenesis. Although the recently described fusion of nucleophosmin to the cytoplasmic tyrosine kinase domain of a transmembrane receptor in chromosomal translocations in some lymphomas is intriguing in this regard, nucleophosmin's proposed role is to provide an active promoter for the kinase (7, 29). Together with the activated expression of *Fgf-8* shown here and the reported transforming potential of *Fgf-8b* cDNA in NIH 3T3 cells (25, 26a), it is likely that *Fgf-8*, and not *Nub-1*, is the gene pertinent to oncogenesis in this locus.

Alternative splicing and gene expression. The transcribed portion of the *Fgf-8* gene is divided into at least five coding exons (Fig. 5A), two more than are found in the other FGF genes examined (1, 14, 19, 24, 28, 49, 50). The first three exons of *Fgf-8* described here correspond to the first exon of other FGF genes. *Fgf-8* is also unusual in that it can be alternatively spliced between exons 2 and 3 to yield at least three protein isoforms which differ only in the amino-terminal portion of the secreted mature protein. We isolated the two previously described cDNAs (42), which code for the isoforms FGF-8b and FGF-8c, and a third cDNA that encodes the previously undescribed isoform FGF-8a (Fig. 5A to C). All three mRNA species were detected in both testis and tumor 61. We have not detected a fourth possible splice variant which would contain exons 2B and 3B in either testis or mammary tumor RNA (data not shown). These cDNA clones will allow a comparison of the three isoforms for possible differences in, for example, their transforming potentials, tissue expression patterns, half-lives, or affinities for the various FGF receptors.

We found expression of *Fgf-8* in adult testis and ovary (Fig. 6), but this expression is much lower than in the tumors with MMTV insertions near *Fgf-8* (Fig. 4). The expression of *Fgf-8* during murine embryogenesis (Fig. 6) suggests that this gene may be important in embryonic development. Recent studies using *in situ* analysis of whole-mount embryos and embryo sections confirm the expression of *Fgf-8* during murine embryogenesis (22). Definitive proof that *Fgf-8* expression is important in murine development will require the creation and analysis of mice lacking a functional *Fgf-8* gene.

The MMTV LTR contains androgen-responsive enhancer elements which can place nearby promoters under androgen regulation (8, 13, 32). In light of our finding that *Fgf-8* is active only in mammary tumors with MMTV proviruses in this locus,

and given that androgen induction of *Fgf-8* has only been demonstrated in cells from a mouse mammary tumor (Shionogi carcinoma 115) and a derivative cell line (SC-3) (42), it seems possible that the androgen-inducible nature of *Fgf-8* in these cells might be attributed to MMTV insertional activation of *Fgf-8* rather than to any inherent feature of the gene. Consistent with this idea is the finding that glucocorticoids—well known for their stimulatory effects on transcription from the MMTV LTR—can also induce *Fgf-8* activity in these cells (47).

***Wnt-1* and *Fgf-8* cooperation.** *Fgf-8* is the third member of the FGF family to be identified as a *Wnt-1* collaborator in mammary carcinogenesis. Surprisingly, only FGF genes have been found to be activated by MMTV in this transgenic model system (37). Assuming that many proto-oncogenes are potential targets for activating insertion mutations, this selectively suggests that FGFs cooperate oncogenically with *Wnt-1* in an especially potent manner. It is possible that the signaling pathways of these two families function in a highly complementary fashion to deregulate mammary epithelial cell growth and proliferation. Alternatively, activated FGFs could contribute to neoplasia in a less direct manner, for example, by stimulating angiogenesis (17, 23). However, it is notable that genes of the *Wnt* and FGF families can evidently collaborate in an experimental model of mesoderm induction (10), suggesting that cooperation in tumorigenesis may derive from the normal interactions of these genes.

The detection of a third activated FGF gene in these tumors prompted us to survey all other known FGF loci for insertions by Southern blot analysis, but no rearrangements in other FGF genes have been found thus far (40). Given the propensity of this model system to activate FGF genes, we speculate that analyses of other common insertion loci in the remaining tumors from this collection may reveal new members of this gene family or identify important downstream components in the FGF signal transduction pathway.

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