JOURNAL OF VIROLOGY, Mar. 1995, p. 1932–1938 Vol. 69, No. 3
0022-538X/95/$04.00+0

Int-6, a Highly Conserved, Widely Expressed Gene, Is Mutated by Mouse Mammary Tumor Virus in Mammary Preneoplasia

ANTONIO MARCHETTI, FIAMMA BUTTITTA, SHUKICHI MIYAZAKI, DANIEL GALLAHAN, GILBERT H. SMITH, AND ROBERT CALLAHAN*

Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Maryland 20892

Received 18 August 1994/Accepted 18 November 1994

With a unique mouse mammary tumor model system in which mouse mammary tumor virus (MMTV) insertional mutations can be detected during progression from preneoplasia to frank malignancy, including metastasis, we have discovered a new common integration site (designated *Int-6*) for MMTV in mouse mammary tumors. MMTV was integrated into *Int-6* in a mammary hyperplastic outgrowth line, its tumors and metastases, and two independent mammary tumors arising in unrelated mice. The *Int-6* gene is ubiquitously expressed as a 1.4-kb RNA species in adult tissues and is detected beginning at day 8 of embryonic development. The nucleotide sequence of *Int-6* is unrelated to any of the known genes in the GenBank database. MMTV integrates within introns of the gene in the opposite transcriptional orientation. In each tumor tested, this results in the expression of a truncated *Int-6*/long terminal repeat (LTR) chimeric RNA species which is terminated at a cryptic termination signal in the MMTV LTR. Since the nonrearranged *Int-6* alleles in these tumors contain no mutations, we favor the conclusion that truncation of the *Int-6* gene product either biologically activates its function or represents a dominant-negative mutation.

The mouse mammary tumor virus (MMTV) has been shown to act as an insertional mutagen that causes the deregulation of expression of adjacent cellular genes in mammary tumors (23). MMTV-infected mice frequently develop preneoplastic hyperplastic alveolar nodules (3, 4, 13, 21). When these are passaged by serial outgrowth in cleared mammary fat pads of syngeneic mice, mammary tumors frequently develop within them in a stochastic manner. In addition, it is not uncommon to find metastatic lesions in the lungs of mice bearing outgrowths with mammary tumors (our unpublished data). This provides a potential experimental approach to dissect, at the molecular level, the virus-induced mutational events that may contribute to different stages of tumor development.

To identify affected cellular genes, the viral genome has been used as a molecular tag. By this approach, five loci (*Wnt-1*/*Int-1*, *Fgf-3*/*Int-2*, *Int-3*, *Wnt-3*, and *Fgf-4*/*Hst*/*k*-FGF) which represent common integration sites (designated *Int* loci) for MMTV in mouse mammary tumors have been identified (6, 7, 15, 17). Except for *Int-3*, the mechanism by which MMTV activates the expression of the *Int* genes is primarily a consequence of the effect of enhancer sequences within the long terminal repeat (LTR) of the integrated MMTV proviral genome on the transcriptional promoter of the adjacent affected gene. In the case of *Int-3*, however, the locus was defined by the integration of an MMTV proviral genome within a 500-bp region that encodes the transmembrane domain of the gene product in the cellular genome of five independent mammary tumors (20). This results in the overexpression of a truncated version of the *Int-3* gene product which corresponds to the intracellular domain of the protein. The thesis that activation of expression of these genes contributes to mammary tumorigenesis has been confirmed in transgenic mouse models in which the transgene corresponds to the MMTV LTR linked to either the *Wnt-1*, *Fgf-3*, or *Int-3* gene (11, 14, 22).

With the exception of *Int-3*, all of the genes activated by MMTV were first identified in mammary tumors arising in

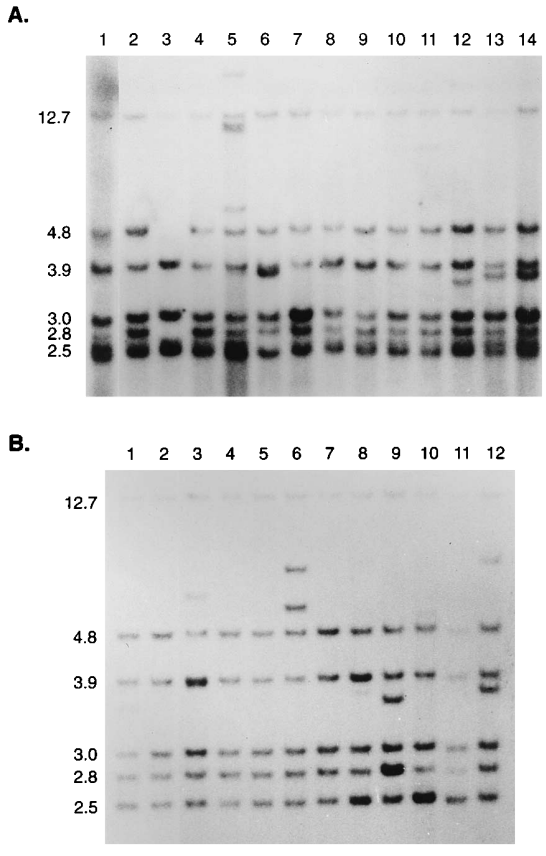

FIG. 1. MMTV proviral genomes in the CZZ-1 preneoplastic HOG cell line and CZZ-1-derived malignant tumors and lung metastasis. CZZ-1 HOG and tumor DNAs (10 μg of each) were digested with *Eco*RI, run on a 0.8% agarose gel, and transferred to a nylon membrane (7). (A) Cellular DNAs were from the CZZ-1 HOG (lane 1) and CZZ-1-derived mammary tumors 22 (lane 2), 1262 (lane 3), 1263 (lane 4), 20 (lane 5), 19 (lane 6), 23 (lane 7), 21 (lane 8), 24 (lane 9), 8 (lane 10), 9 (lane 11), 12 (lane 12), 13 (lane 13), and 14 (lane 14). (B) Cellular DNAs were from tumor 4973 (lane 1) and 11 independent lung metastases (lanes 2 to 12) from the mouse bearing tumor 4973. The membranes were hybridized with the MMTV LTR probe at 65°C under previously described conditions (7).

* Corresponding author.

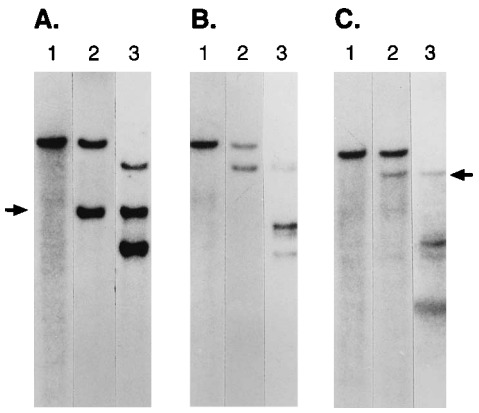

FIG. 2. Identification of the *Int-6* common integration site in the CZZ-1 HOG-derived tumor 22 (A, lanes 2 and 3) and independent mammary tumors 1139 (B, lanes 2 and 3) and 3144 (C, lanes 2 and 3). Cellular DNAs (10 μg) from each tumor DNA and normal liver DNA (lane 1 in each panel) were digested with *Eco*RI, run on a 0.8% agarose gel, and then blotted onto a nylon membrane. The blots for panels A and B were sequentially hybridized with probes corresponding to the host flanking sequences, probe D (Fig. 3) (lanes 1 and 2), and MMTV *gag* sequences (lane 3). In panel C, the blot was sequentially hybridized with probe C (Fig. 3) (lanes 1 and 2) and MMTV *env* sequences (7) (lane 3). Arrows indicate the MMTV-induced rearranged restriction fragments.

inbred mouse strains which had been bred for 40 to 50 years for a high incidence of mammary tumors. Published reports indicate that the frequency with which *Wnt-1* and *Fgf-3* are rearranged in MMTV-induced mouse mammary tumors varies significantly between different high-incidence inbred mouse strains (reviewed in reference 16). To identify new *Int* loci in mammary tumor DNAs that would not be biased by selective inbreeding for a high-cancer phenotype, we have focused on a feral mouse strain derived from a single breeding pair of *Mus musculus musculus* trapped in Czechoslovakia (CZECH II) (1, 7). The CZECH II mice, unlike high-incidence inbred mouse strains, lack endogenous or genetically transmitted MMTV genomes but do contain an infectious strain of MMTV that is transmitted congenitally through the milk. In the CZECH II mice, we have developed several preneoplastic hyperplastic outgrowth lines (HOGs) which are stable clone-dominant populations and develop focal mammary tumors which sometimes metastasize to the lung (21a). In this report, we describe a new *Int* gene (designated *Int-6*) which was first detected in one of these CZECH II HOGs.

DNA from one CZECH II HOG, designated CZZ-1, was found by Southern blot analysis of *Eco*RI-digested cellular DNA to contain three MMTV proviral genomes (six *Eco*RI restriction fragments) integrated into the cellular DNA (Fig. 1). CZZ-1 proved to be a clone-dominant population of preneoplastic cells, because these six MMTV-related fragments were reproducibly found in the original outgrowth and in each succeeding transplant generation. These proviral genomes were also present in primary tumors which arose independently from within the HOG (Fig. 1A). The clone-dominant nature of the CZZ-1 HOG is illustrated by tumor 1262 (lane 3), in which one complete proviral genome, corresponding to the 4.8- and 2.8-kb *Eco*RI restriction fragments, has been lost. Five of 13 of the tumor DNAs (lanes 5, 11, 12, 13, and 14), however, contained additional integrated MMTV proviral genomes. Similarly, 5 of 11 independent metastatic lesions in the lung of the mouse bearing the *Int-6*-positive tumor 4973 contained additional integrated MMTV genomes (Fig. 1B, lanes 3, 6, 8, 9, and 12). These results raised the possibility that the additional integration events may have contributed to tumor progression by activating (or inactivating) additional cellular genes.

Since we found that none of the known common insertion sites (*Wnt-1*, *Fgf-3*, *Int-3*, *Wnt-3*, and *Fgf-4*) were rearranged by MMTV in the CZZ-1 HOG (data not shown), recombinant clones were obtained for each of the *Eco*RI host-MMTV junction restriction fragments. Subclones of the host sequences were used as probes to screen Southern blots of independent MMTV-induced mammary tumor DNAs for evidence of virus-induced DNA rearrangements. With this approach, a probe consisting of the host sequences flanking one of the MMTV proviruses (3.0-kb fragment, Fig. 1) in the CZZ-1 HOG detected MMTV-induced rearrangements in 2 of 20 additional independent MMTV-induced mammary tumor DNAs (Fig. 2). In each case, the rearranged restriction fragment comigrated with a fragment containing either MMTV *gag* or *env* sequences. From these data, we were able to deduce that the transcriptional orientation of the viral genome in each tumor was in the same direction. Thus, the host sequences adjacent to the integrated MMTV genome define a new common integration site, which we have named *Int-6*.

To obtain recombinant genomic clones of the *Int-6* locus, we have used a subclone of the 3.0-kb *Eco*RI fragment (Fig. 1) containing the host flanking sequences (probe D, Fig. 3) to probe a phage lambda library of mouse genomic DNA. An additional three overlapping lambda clones which span 47 kb of the *Int-6* locus have also been obtained by using probes A to C (Fig. 3). These probes were also hybridized to Northern (RNA) blots of tumor RNAs to detect the expression of the putative target gene in the *Int-6* locus. Probe A detected a 1.4-kb species of RNA in each of 10 tumors tested (data not shown). A Northern blot of three of these tumor RNAs is shown in Fig. 4. Two of the tumors (22, derived from CZZ1 HOG, and 1139) had viral insertions in *Int-6*. In addition, tumor 1139 also contained a 0.9-kb species of RNA related to sequences in probe A. However, tumor 178 (Fig. 4) and several other MMTV-induced mammary tumors in which *Int-6* is not rearranged by the virus also express the 1.4-kb RNA species detected by probe A (data not shown). This suggests that in tumors 22 and 1139, the level of *Int-6* gene expression is not an important consequence of viral integration in this locus. Tissue from tumor 3144 (Fig. 3) has been exhausted and therefore could not be further studied.

We have used probe A to obtain cDNA clones of the 1.4-kb *Int-6* RNA species from a cDNA library of an MMTV-induced tumor in which *Int-6* was not rearranged by MMTV. The nucleotide sequence corresponding to this species of *Int-6* RNA was determined and is shown in Fig. 5. Further nucleotide sequence analysis of the genomic clones of the *Int-6* locus demonstrated that this RNA species is encoded by 13 exons which span 34 kb of genomic DNA (Fig. 3). Translation of the 1.4-kb species of *Int-6* RNA revealed an open reading frame which could encode a 50.2-kDa protein. This protein contains two potential N-glycosylation site motifs (8) as well as potential phosphorylation site motifs for cyclic AMP/cyclic GMP-dependent protein kinase (9), protein kinase C (12), tyrosine kinases (10), and casein kinase II (18) (Fig. 5). However, a comparison of the *Int-6* nucleotide sequence and the amino acid sequence of the translated protein with sequences of known genes in the GenBank database showed that it is unrelated to any of these genes or their corresponding gene products.

All of the genes whose expression has been affected or altered by MMTV integration in mouse mammary tumors have been highly conserved through evolution (5, 19, 20). We have surveyed by Southern blot analysis the conservation

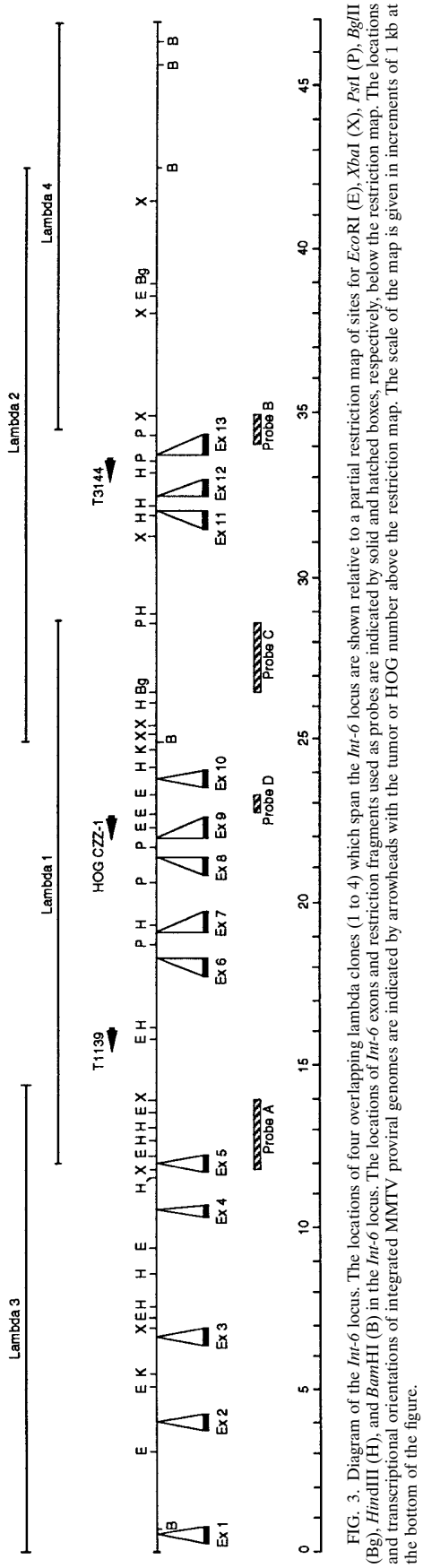

FIG. 3. Diagram of the *Int-6* locus. The locations of four overlapping lambda clones (1 to 4) which span the *Int-6* locus are shown relative to a partial restriction map of sites for *Eco*RI (E), *Xba*I (X), *Ps*I (P), *Bgl*II (Bg), *Hin*dIII (H), and *Bam*HI (B) in the *Int-6* locus. The locations of *Int-6* exons and restriction fragments used as probes are indicated by solid and hatched boxes, respectively, below the restriction map. The locations and transcriptional orientations of integrated MMTV proviral genomes are indicated by arrowheads with the tumor or HOG number above the restriction map. The scale of the map is given in increments of 1 kb at the bottom of the figure.

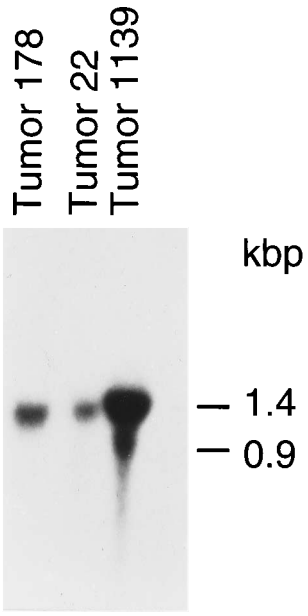

FIG. 4. *Int-6* expression in CZECH II mammary tumors. Total RNA (20 μg) prepared from the *Int-6*-negative tumor 178 and the *Int-6*-positive tumors 22 and 1139 was denatured in the presence of formaldehyde and run on a 1% agarose gel containing formaldehyde. The RNA was transferred to a nylon membrane and hybridized (7) with probe A (Fig. 3).

of the *Int-6* gene in genomic DNA from different species. As shown in Fig. 6, *Int-6*-related sequences can be detected in all eukaryotic species of cellular DNA tested, including human, chicken, *Xenopus*, *Drosophila*, and *Caenorhabditis elegans* cells.

Having detected the 1.4-kb *Int-6* RNA species in a tumor in which the gene was not rearranged by MMTV, we surveyed normal adult tissues and embryos at different stages of development for *Int-6* RNA by Northern blot analysis. *Int-6* RNA is present in various amounts in all adult tissues which we have tested, including the mammary gland (Fig. 7). In addition, we could detect *Int-6* RNA in embryos as early as day 8 of development.

What then are the consequences of MMTV integration into the *Int-6* gene? Inspection of our mapping data, illustrated in Fig. 3, shows that all of the viral integration events occur within introns of the *Int-6* gene and that the transcriptional direction of the integrated viral genome is in the opposite orientation from that of the *Int-6* gene. There are at least four possible scenarios for the role that MMTV plays at this locus that are compatible with our current findings. In the first, the *Int-6* gene which we have identified is in fact not the target gene on which MMTV acts. Consider the organization of the *Wnt-1*, *Fgf-3*, and *Fgf-4* loci (6, 15, 17). At these loci, MMTV integration sites cluster around the target gene in an ordered fashion. The transcriptional orientation of the integrated viral genomes 5′ of the target gene is in the opposite direction from that of the target gene, whereas those 3′ of the target gene are in the same transcription orientation. From published reports and our own experience, MMTV integration sites are within 15 kb of the particular target gene, if enhancer activation of the target gene promoter is involved. Since we have found no evidence for the activation of expression of RNA corresponding to sequences up to 13 kb 3′ of the *Int-6* gene, the location of the putative target gene would have to be more than 24 kb from the inte-

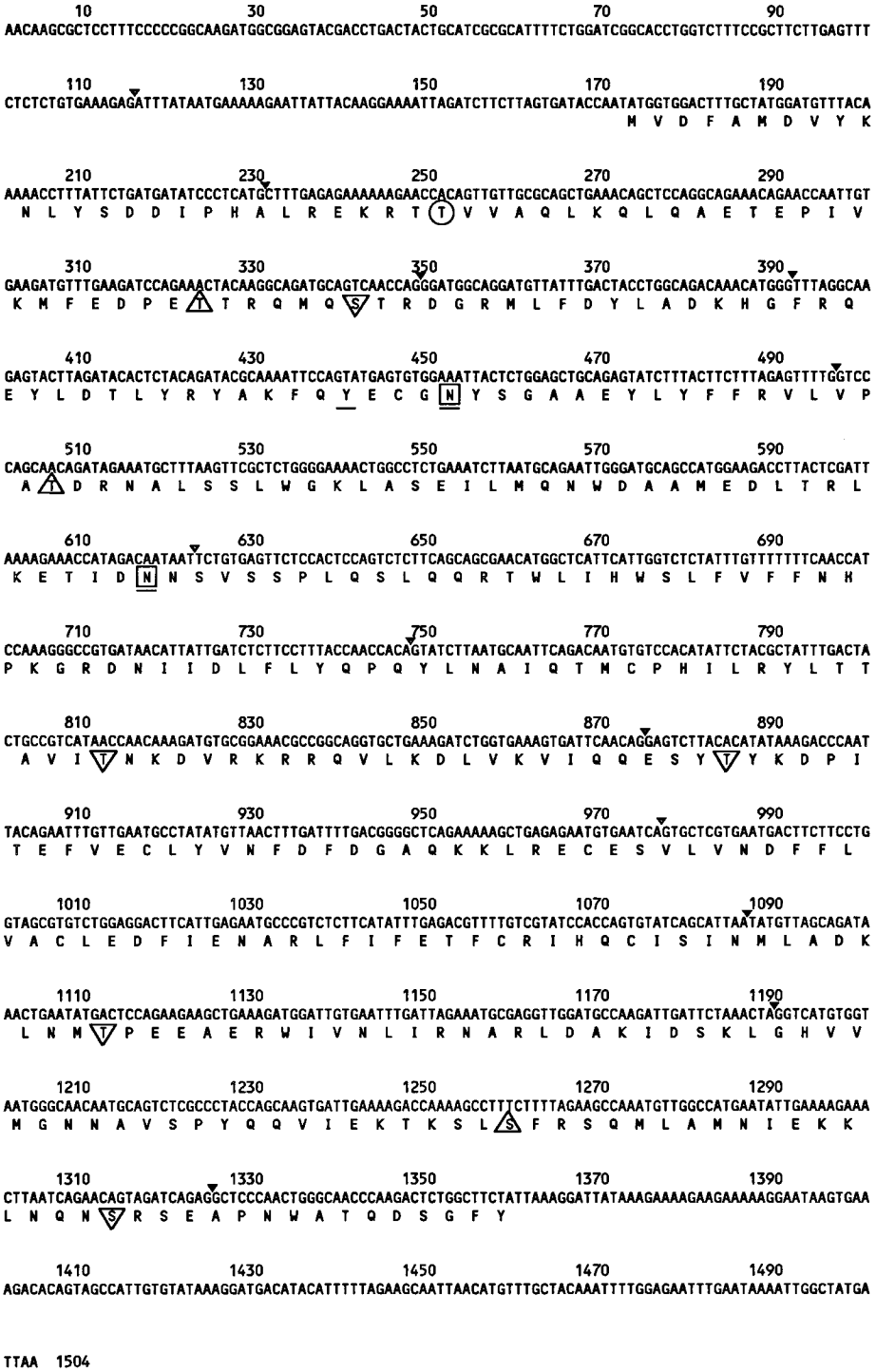

FIG. 5. Complete nucleotide sequence of the 1.4-kb *Int-6* RNA transcript. The intron breaks are indicated by small arrowheads above the start of the next exon. The deduced amino acid sequence of the gene product is given below the nucleotide sequence. Potential phosphorylation sites for cyclic AMP/cyclic GMP-dependent protein kinase (○), protein kinase C (△), tyrosine kinases (——), casein kinase II (▽), and glycosylation sites (□) are indicated. Abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

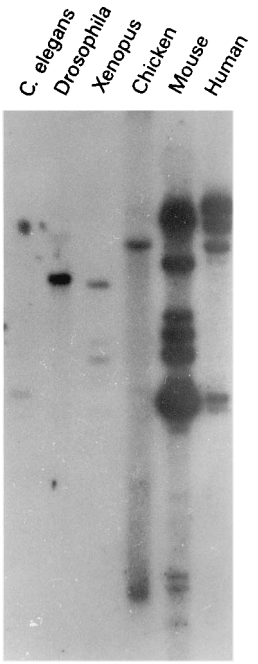

FIG. 6. Conservation of *Int-6* nucleotide sequences among eukaryotic species. Cellular DNAs (10 μg each) from *C. elegans*, *Drosophila*, *Xenopus*, chicken, mouse, and human cells were digested with *Bam*HI, run on a 0.8% agarose gel, and transferred to a nylon membrane. The blot was hybridized with *Int-6* cDNA in 2× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4 [pH 7.4], and 1 mM EDTA)–5× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin)–5% dextran sulfate–2% sodium dodecyl sulfate (SDS) at 65°C. After hybridization, the blot was washed in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS at 65°C. Exposure to Kodak XAR-5 film was for 3 days.

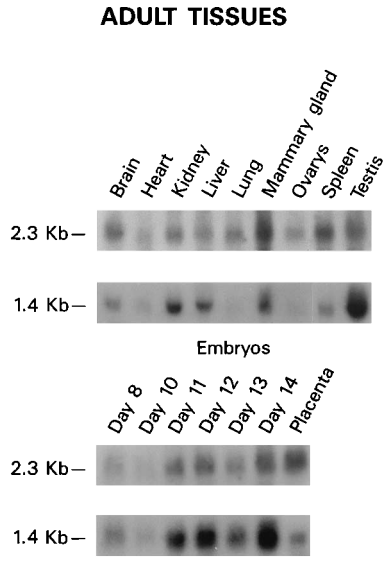

FIG. 7. *Int-6* RNA expression in normal adult tissues and developing embryos. Total RNAs (10 μg each) were prepared from the indicated tissues. The RNAs were denatured in the presence of formaldehyde and run on 1% agarose gels containing formaldehyde. The RNA samples were then transferred to a nylon membrane and hybridized sequentially with a β-actin probe and then an *Int-6* cDNA probe.

grated MMTV genome in CZZ-1 and 30 kb in tumor 1139 (Fig. 3).

A second possible consequence of MMTV integration into the *Int-6* gene is the activation of expression of a new chimeric RNA transcript of *Int-6* initiated by the 3′ LTR of the integrated viral genome. An analogous situation occurs in MMTV-induced rearrangements of *Int-3* (20), except that in the case of *Int-6* this would result in the expression of *Int-6* antisense RNA, since the transcriptional orientation of the viral genome is opposite that of *Int-6*. This would represent a *trans*-dominant mutation that would inactivate the expression of both alleles. However, using a variety of techniques, we have not detected MMTV-induced *Int-6* antisense RNA expression (data not shown).

A third possibility assumes that the gene that we have identified is the target for MMTV integration within the *Int-6* locus. In this case, the viral insertion would disrupt the expression of one allele and reveal the presence of a spontaneous recessive mutation in the other allele. To examine this possibility, we determined the nucleotide sequence of cDNA corresponding to the nonrearranged allele of *Int-6* in tumor 22 and tumor 1139 (data not shown). In both cases, no mutation was found.

The fourth and more plausible scenario is that MMTV integration into the *Int-6* gene causes the expression of a biologically activated gene product (like *Int-3* [20]) or a dominant-negative gene product, either of which deregulates the normal control of mammary epithelial cell growth, leading to hyperplasia of the affected mammary epithelial cells. This would create a premalignant epithelial cell population within which mammary tumors could subsequently develop. We have determined the nucleotide sequence of cDNA clones of *Int-6* RNA from tumor 1139 and tumor 22. In each case, transcription of the rearranged allele resulted in the expression of a chimeric RNA species which terminated at a cryptic transcription stop signal in the reverse U3 portion of the MMTV LTR (Fig. 8A). A similar cryptic transcription termination signal has previously been shown to be active in certain MMTV-induced rearrangements of *Fgf-3* (2). In tumor 1139, two RNA species from the rearranged allele (900 and 965 bp) were detected (Fig. 8B). They correspond to the 900-bp RNA species detected by Northern blot analysis (Fig. 4). In one RNA species, exon 5 was spliced to the end of the U5 portion of the MMTV LTR, and in the other species, splicing occurred at a cryptic splice acceptor site in intron 5. Similarly, in tumor 22, there were three chimeric RNA species in which exon 9 was spliced to one of three different cryptic splice acceptor sites in intron 9. Since the size of the chimeric tumor RNA species is similar to that of the normal *Int-6* RNA, they went undetected in the Northern blot analysis of tumor 22 RNA (Fig. 4).

Translation of the rearranged *Int-6* RNA species into putative proteins revealed that from all five species, the product is a truncated chimera of the *Int-6* protein and is composed of normal *Int-6* amino acid sequences linked to novel amino acid sequences encoded by an *Int-6* intron and/or reverse MMTV LTR nucleotide sequences. Whether these novel sequences are important for the biological consequences of MMTV integration into this gene is unknown at present. However, the 965-bp RNA species in tumor 1139 suggests that truncation of the *Int-6* gene product is the more important consequence. In this RNA species, only a single amino acid is added to the truncated *Int-6* gene product. Currently, tests are being developed to determine the biological activity of the MMTV-induced rearranged gene products.

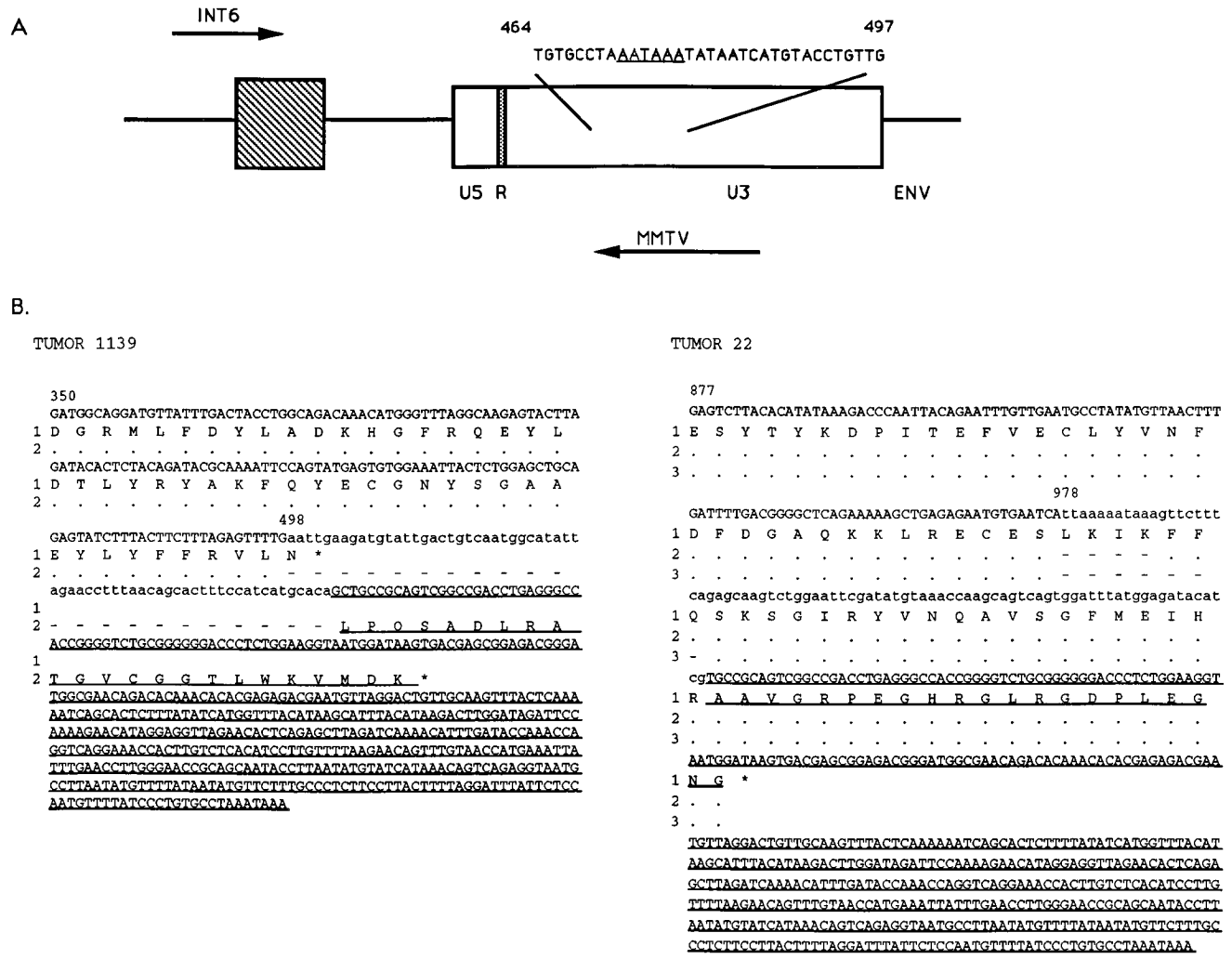

FIG. 8. Rearrangement of *Int-6* by MMTV leads to the expression of novel species of *Int-6* RNA in mammary tumors. (A) Location and nucleotide sequence of a cryptic transcription stop signal (underlined) in the reverse sequence of the MMTV LTR. The reverse sequence shown corresponds to bases 464 through 497 from the 3′ end of the LTR. The hatched box corresponds to an *Int-6* exon. The open box corresponds to the LTR of an MMTV proviral genome integrated within the *Int-6* gene. The U5, R, and U3 regions of the MMTV LTR are indicated, as well as the transcriptional orientation of the MMTV genome and *Int-6*. (B) The nucleotide sequence of the junction between MMTV and *Int-6* sequences in chimeric RNA species detected in tumors 22 and 1139. In tumor 1139, the nucleotide sequence shown begins at the 5′ end of exon 4. The nucleotide sequence in lowercase letters corresponds to intron 5 sequences. Nucleotide and amino acid sequences which are underlined are from the integrated MMTV genome. In RNA species 2, nucleotide sequences which have been spliced out are indicated by dashes. Amino acid sequences in RNA species 2 which are identical to those in RNA species 1 are indicated by dots. The nucleotide sequence shown for the chimeric RNA species detected in tumor 22 begins at the 5′ end of exon 9 through a portion of intron 9 to the cryptic poly(A) addition signal in the MMTV genome. The intron 9 nucleotide sequence is given in lowercase letters. MMTV sequences are underlined. Dashes indicate regions of the intron which have been spliced out. Dots correspond to amino acid residues encoded by RNA species 2 and 3 which are identical to those encoded by RNA species 1. The abbreviations for amino acids are the same as in the legend to Fig. 5.

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