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Wnt-3, a gene activated by proviral insertion in mouse mammary tumors, is homologous to *int-1/Wnt-1* and is normally expressed in mouse embryos and adult brain

(provirus tagging/embryogenesis/*int-1/Wnt-1* gene family)

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ABSTRACT **We have isolated a common insertion site, *Wnt-3*, for proviruses of the mouse mammary tumor virus (MMTV). Of mammary tumors induced by the GR variant of MMTV, 5% contains a provirus at *Wnt-3*, which is located on mouse chromosome 11. The gene is transcribed into a 3.8-kilobase (kb) mRNA in tumors with nearby proviral insertions but not in tumors with proviruses at other loci or in most adult tissues. Normal expression of *Wnt-3* is detected in mouse embryos (with a peak around day 12 of gestation) and at low levels in adult brain. The transcriptional unit of the *Wnt-3* gene spans ≈55 kb, with a first intron of 36 kb. The deduced amino acid sequence of the Wnt-3 protein is 47% identical to the *int-1/Wnt-1* gene product.**

Proviral insertion near and activation of host-cell oncogenes is an important step in mammary tumorigenesis by the mouse mammary tumor virus (MMTV). Most of the genes at MMTV insertion sites, collectively called *int*, were originally isolated by provirus tagging, with the inserted MMTV provirus as a starting point for molecular cloning (reviewed in ref. 1). Two of these genes, *int-1* and *int-2*, have been analyzed in great detail. (Recently, a new nomenclature has been proposed for *int-1* and genes related to *int-1* by sequence homology. *int-1* is now called *Wnt-1*; and the *irp* gene is called *Wnt-2*.)

Although *Wnt-1* and *int-2* are not structurally related to each other, they share two intriguing properties: temporal expression during early development of the mouse, in a very restricted pattern; and protein products with characteristics of secreted factors (reviewed in ref. 2). *Wnt-1* is the mouse homolog of the *Drosophila* segment polarity gene wingless (3); *int-2* is a member of the fibroblast growth factor family (4). The *int-3* gene has not been analyzed at the molecular level, but it has a very restricted expression pattern as well (5). Thus, it appears that MMTV proviral insertion leads to tumorigenesis by transcriptional activation of genes that are silent in normal mammary cells or in most other adult tissues. The isolation of more of these common integration sites may tell whether this is a general phenomenon and may yield novel genes involved in the control of embryogenesis.

The frequency of activation of the different *int* genes varies considerably between mouse strains. In the C3H and the BR6 strains, for example, nearly all tumors have an activated *Wnt-1* or *int-2* allele (6, 7), but in the GR strain many tumors are negative for insertion at any of the known loci (8). Therefore, we have searched for new *int* loci in GR virus-induced mammary tumors. Here we present the identification of a previously unreported common integration area, *Wnt-3*.† We find *Wnt-3* expression during embryogenesis, in adult mouse brain, and in differentiating P19 embryonal carcinoma cells. The sequence of *Wnt-3* shows homology to that of *Wnt-1*.

MATERIALS AND METHODS

Biological Materials. Newborn BALB/c mice were foster-nursed on GR mothers. These mice are referred to as BALB/cfGR. Breeding GR and BALB/cfGR mice were examined for the presence of spontaneous mammary tumors. When occurring, these tumors were excised from the mice. Tumor TSL81 was a transplanted tumor from a GR mouse, described earlier (8) and kindly provided by M. Sluyser. The somatic cell hybrids used were provided by J. Hilkens and have been described (9). Tissue for nucleic acid extraction was frozen in liquid nitrogen and stored at −70°C.

Embryos were excised from pregnant BALB/c or GR mice. Appearance of the vaginal plug was denoted as day 0.5 of gestation. Embryos were frozen in liquid nitrogen and stored at −70°C.

Isolation and Analysis of High Molecular Mass Cellular DNA. Frozen tissue was minced using scissors. The tissue was digested for 16 hr at 56°C with 0.1 mg of proteinase K per ml in TE buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA) containing 0.1% sodium dodecyl sulfate (SDS). The nucleic acids were extracted twice with an equal volume of 1:1 (vol/vol) phenol/chloroform. After precipitation with ethanol, the DNA was spooled on a glass rod and dissolved in TE buffer. RNA was hydrolyzed for 1 hr at 37°C with 10 μg of RNase A per ml. Phenol/chloroform extractions were repeated, and after precipitation the DNA was dissolved in TE buffer and stored at 4°C. DNAs were digested overnight in buffers recommended by the suppliers. Usually 20 μg of DNA was digested with a 5-fold excess of restriction enzymes, electrophoresed on 0.8% agarose gels, and transferred to nitrocellulose filters. The filters were hybridized for 12–16 hr at 65°C with ^{32}P-labeled probes in 3× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% SDS, 50 μg of denatured salmon sperm DNA per ml, and 10% dextran sulfate. The filters were washed at 65°C in 0.1× SSC/0.1% SDS and exposed to XS or XAR-5 film at −70°C with intensifying screens.

Molecular Cloning of Viral and Host Junction Sequences. The majority of techniques for isolation and purification of molecular clones were as described (10). DNA from tumor BG62 was digested with *Eco*RI, and 21-kilobase (kb) fragments were isolated by using sucrose gradient centrifugation. These fragments were ligated into *Eco*RI-digested phage

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Abbreviations: MMTV, mouse mammary tumor virus; PCR, polymerase chain reaction.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M32502).

λEMBL4. After packaging, the recombinant phages were plated on *Escherichia coli* VCS 257 and screened by previously described procedures (10).

Culturing of P19 Embryonal Carcinoma Cells. P19 cells were grown in 1:1 (vol/vol) Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium with 5% fetal calf serum in an incubator at 37°C with an atmosphere of 5% CO_2/95% air and 100% humidity. To induce differentiation to neural cells, the cells were grown for 6 days as aggregates (or embryoid bodies) in bacterial-grade Petri dishes in the presence or absence of 0.1 μM retinoic acid. The embryoid bodies subsequently were transferred to tissue culture-grade Petri dishes and were kept in culture in the presence or absence of 0.1 μM retinoic acid. Cells were collected at several time points for RNA isolation. Day 0 is the onset of growth as embryoid bodies.

Isolation of Total Cellular RNA. Frozen tissue or cultured cells were homogenized in 3 M LiCl/6 M urea with a Polytron homogenizer. RNA was precipitated overnight at 0°C and pelleted by centrifugation in an SW 27.1 rotor at 20,000 rpm. The pellet was dissolved in TE buffer containing 0.1% SDS and was extracted twice with 1:1 phenol/chloroform. Total cellular RNA was stored as an ethanol precipitate at −20°C.

Gel Analysis of RNA. RNA samples were electrophoresed on gels containing 1% agarose in 1× RNA running buffer (20 mM 4-morpholinepropanesulfonic acid/5 mM sodium acetate/5 mM EDTA/2.2 M formaldehyde). Total cellular RNA (20 μg) was pelleted and dried in an Eppendorf tube. The RNA was dissolved in sample buffer containing 50% (vol/vol) formamide, 2.2 M formaldehyde, and RNA running buffer, heated for 10 min at 65°C. Gels were blotted onto a nitrocellulose filter. The RNA filters were hybridized, washed, and autoradiographed under identical conditions to those used for DNA hybridization and autoradiography.

RNase Protection Experiments. Solution hybridization and RNase mapping were performed essentially as described (11).

cDNA Cloning and Sequencing. Plaques (5×10^6) of a cDNA library prepared from RNA from a day 8.5 mouse embryo were screened by standard techniques with probe pBG87. Plaques (10^6) of the same library were screened with probes pBG42 and pBG81. Hybridizing phage DNA inserts were subcloned in pGEM-3Zf(+) and sequenced by using primers specific for the phage SP6 and T7 promoters as well as designed primers internally hybridizing to cloned sequences. All sequence reactions were performed on denatured double-stranded templates by using Sequenase version 2 according to the manufacturer's instructions (United States Biochemical). Both strands were sequenced.

Polymerase Chain Reaction (PCR). First-strand cDNA synthesis was performed by standard techniques (12) with 0.5 μg of poly(A)$^+$ RNA from a day 13.5 mouse embryo. Synthesis was primed with the oligonucleotide CCCCGGAACTGATGCTGG (positions 334–351 in the cDNA sequence; see Fig. 4) derived from the (−)-strand. The cDNA reaction mixture was diluted 1:50 in 50 μl of the PCR reaction mixture according to Perkin–Elmer/Cetus. Using 150 ng of (+)-strand primer AGTGGACTTTGTTCCAAC (positions 14–31 in the cDNA sequence; see Fig. 4) derived from the sequence of the first exon in pBG42 as well as 150 ng of the same primer used in first-strand cDNA synthesis, we performed 40 cycles (45 sec at 94°C, 2 min at 50°C, 2 min at 72°C) with a dry block temperature cycler. The PCR fragments were directly cloned in *Sma* I-linearized pGEM-3Zf(+) and sequenced.

RESULTS

Cloning of the *Wnt-3* Locus. The identification and molecular cloning of acquired MMTV proviruses in GR mouse mammary tumors is complicated by the multitude of endogenous MMTV copies in the GR strain itself. To simplify the analysis of integration sites, we collected a set of tumors induced by the GR virus variant in BALB/c mice, a strain with few endogenous proviruses. Like the GR mouse tumors, these so-called BALB/cfGR tumors are heterogeneous with respect to activation of the different activated oncogenes; we found rearrangements of the known *int* genes at various frequencies, but many tumors apparently had acquired MMTV proviral DNA at other, not-yet-identified loci.

Tumor BG62 contained a single extra provirus not integrated in *Wnt-1*, *int-2*, or *int-3*. A restriction map of the integration domain in tumor BG62 was assembled and, by using a MMTV *env*-specific probe, a 21-kb *Eco*RI fragment, λBG11, was cloned from a tumor DNA library (Fig. 1). A single-copy DNA fragment, pBG14, derived from the cellular domain was hybridized to *Eco*RI and *Eco*RV digests of ≈80 BALB/cfGR tumors, 20 GR tumors, and 10 C3H tumors. In 2 BALB/cfGR tumors (BG15 and BG55) and 2 GR tumors (GG6 and TSL81), a proviral insertion in the locus was found, showing that we had cloned a new common integration site for MMTV, *Wnt-3*. To obtain clones covering the complete *Wnt-3* locus, we performed a chromosome walk, using genomic libraries of normal mouse DNA. By using various probes on transverse alternating-field electrophoresis gels of *Sac* II-digested normal mouse DNA, we could show that the clones were all derived from the same locus. Fig. 1*A* shows the assembled restriction map of the *Wnt-3* locus. All restriction sites were mapped at corresponding positions, both in normal and in cloned DNA. Point zero on the map is arbitrarily chosen as the integration site of the MMTV provirus cloned from tumor BG62.

Transcriptional Unit at *Wnt-3*. Cloned genomic fragments were screened for the presence of exon sequences by hybridization to blots with RNA from mammary tumors GG6, BG55, and TSL81—all with an MMTV integration at the *Wnt-3* locus—and from normal tissues. Two separate areas were located from which fragments hybridized to a mRNA of 3.8 kb in the three tumors (Fig. 1*A*; data on TSL81 not shown). These areas were represented by probes pBG42 and pBG81 (Fig. 1*B*). With probe pBG81, the normal expression pattern of *Wnt-3* was determined. We did not find *Wnt-3* expression in any tumor with a proviral integration at other *int* loci, as exemplified by tumor TSL78 in Fig. 2*A*, or in normal mammary gland (not shown), indicating that *Wnt-3* is activated in mammary tumors because of a nearby insertion. Probes isolated from the other side of the integration cluster did not detect transcripts in tumors.

Besides normal mammary gland, we examined liver, spleen, uterus, placenta, testis, heart, lung, skeletal muscle, and brain from adult mice. *Wnt-3* was expressed, albeit at a low level, in the brain (Fig. 2*B*) and not anywhere else in adult mice (not shown).

We next examined mouse embryos for normal *Wnt-3* expression. We isolated RNA from embryos ranging in age from day 7.5 to day 17.5 postcoitum. Fig. 2 shows that *Wnt-3* expression was found in embryos from day 10.5 postcoitum until the end of gestation. Expression was high around day 12.5 but was still detectable at day 17.5 of gestation (Fig. 2*C*). Our analysis of the *Wnt-3* expression in sectioned embryos using an RNase protection assay showed that the expression was localized in the dorsal side and the head (not shown).

In view of the expression during embryogenesis, we also searched for *Wnt-3* transcripts in embryonal carcinoma cells. We found relatively high levels of *Wnt-3* transcripts in P19 cells treated with retinoic acid (Fig. 2*D*). Expression was only observed when the cells were grown as embryoid bodies in the presence of retinoic acid, which induces neural differentiation in these cells (13).

Structure of the *Wnt-3* Gene. To map the 5′ end of the gene and to determine the orientation of transcription, we used

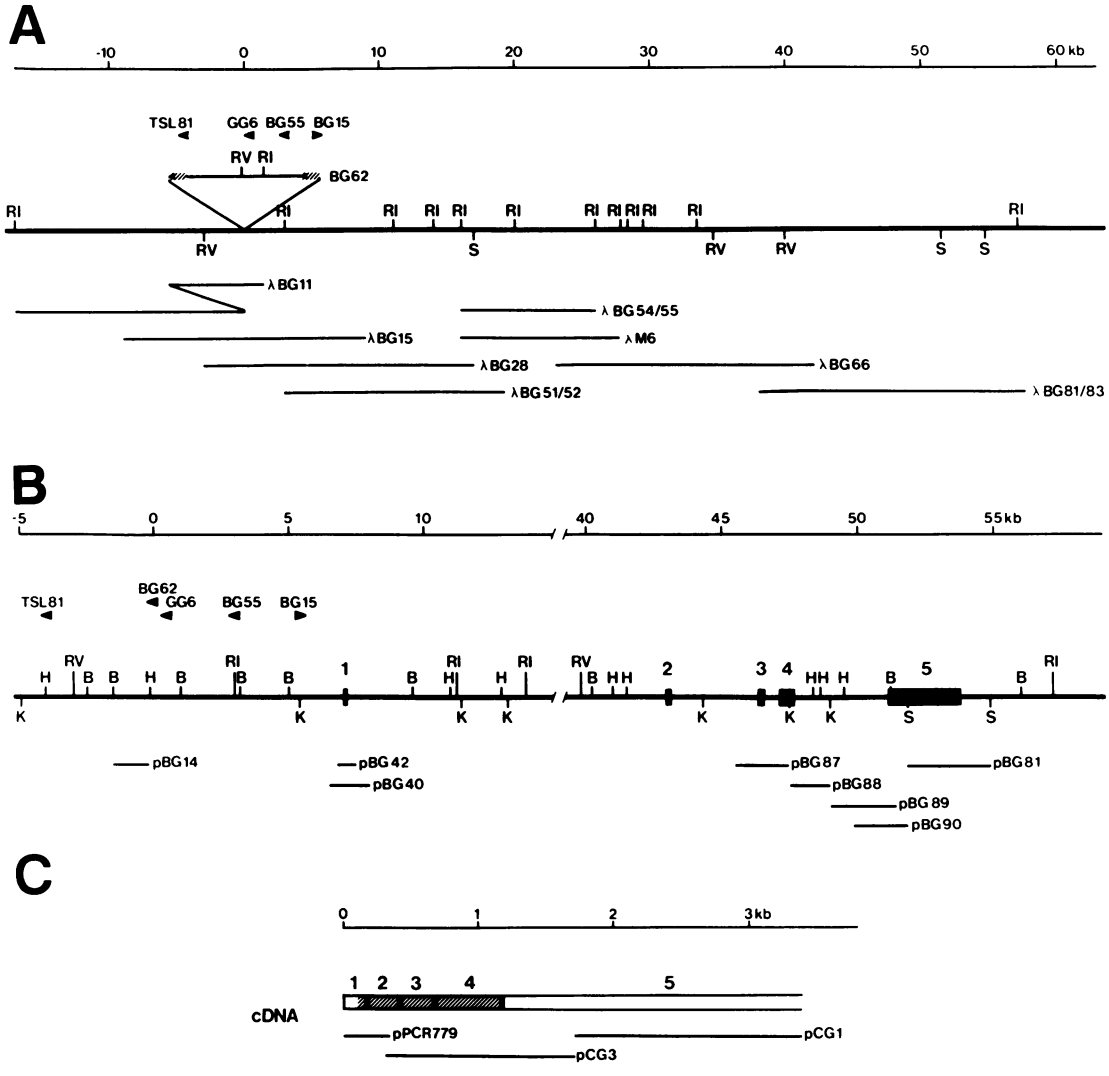

FIG. 1. Restriction map of the *Wnt-3* locus. Arrowheads indicate the site and orientation of MMTV proviral integrations found in the *Wnt-3*-rearranged tumors. Restriction enzyme sites are: B, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV; H, *Hin*dIII; K, *Kpn* I; and S, *Sal* I. The different probes used in hybridization experiments are shown below the restriction map. (*A*) Overall restriction map as well as the phage inserts. (*B*) More detailed map of the regions containing the exons. The positions of the exons are indicated by numbered boxes. (*C*) Wnt-3 cDNA. Indicated are the isolated cDNA clones as well as the fragment generated in the PCR reaction, pPCR779. The hatched block indicates the protein-coding domain. The numbered blocks represent the exons.

RNase protection assays with *in vitro* synthesized labeled RNA (11). Probe pBG42 was subcloned in pSP64 and pSP65, allowing *in vitro* transcription in two orientations. A fragment of 175 nucleotides was protected by RNA from tumors GG6, BG55, and TSL81 and RNA from retinoic acid-treated P19 cells but not by control tRNA, as determined with the probe transcribed from pBG42 cloned in pSP64 (Fig. 3; TSL81 not shown). With an opposite strand probe, no protected fragments were detected (not shown), demonstrating that the transcriptional orientation of the *Wnt-3* gene is from the left to the right on the map in Fig. 1. The orientation was confirmed with RNA probes derived from clones pBG88 and pBG89 (not shown). At the *Wnt-1* and *int-2* loci, most of the integrated proviruses have their transcriptional orientation pointing away from the gene, indicating that activation is the consequence of enhancement from the inserted provirus (6, 14). This configuration appears to be prevalent at *Wnt-3* as well. The provirus in tumor BG15 that is in the same transcriptional orientation as *Wnt-3* could be a promoter insertion, which is also seen rarely at *Wnt-1* and *int-2*.

With probe pBG40, a probe containing more sequences both 5′ and 3′ compared with those of pBG42, the same 175-bp fragment was detected, showing that this represents a complete exon. Probes upstream of pBG42 did not hybridize to tumor RNA, indicating that the 175-bp fragment probably represents the first *Wnt-3* exon. pBG42 was completely sequenced and the position of the 175-nucleotide exon was assigned, based on hybridization of subfragments and the position of a consensus splice donor site.

To obtain cDNA clones from the *Wnt-3* gene, we used probes pBG42, pBG87, and pBG81 to screen a cDNA library prepared from RNA of a day 8.5 total mouse embryo (gift of B. Hogan). No clones were found to hybridize to probe pBG42, but several overlapping cDNA clones were obtained by hybridization to the downstream fragments. These clones were partially or completely sequenced. We found no overlap of this cDNA sequence with the sequence of the *Wnt-3* exon in pBG42. Using a (+)-strand primer in the exon in pBG42 and a (−)-strand primer from the 5′ end of the most-upstream cDNA clone, we performed a PCR (15) on RNA (16) from a day 13.5 mouse embryo. This PCR fragment was subcloned in the *Sma* I site of pGEM-3Zf(+) to generate plasmid pPCR779 and was sequenced. The sequence of the first 161 bases was identical to the sequence of the exon in pBG42.

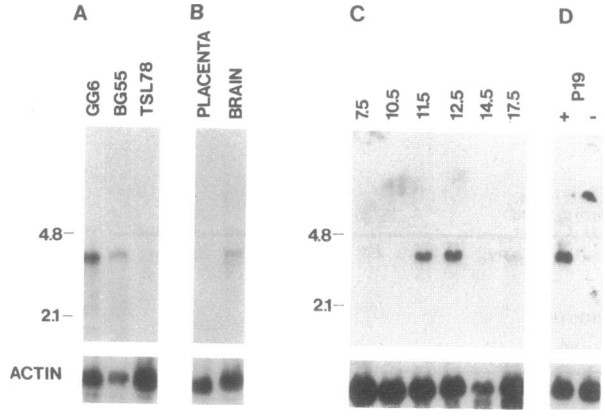

FIG. 2. RNA blots with RNA obtained from various sources and hybridized with pBG81. As a control for the amount and quality of the loaded RNA, a control hybridization with an actin probe is included. (*A*) Hybridization with RNA derived from tumors GG6 and BG55, each harboring a MMTV proviral integration in the *Wnt-3* locus, as well as with RNA from tumor TSL78, not harboring a MMTV integration in the *Wnt-3* locus. (*B*) RNA derived from brain and placenta. (*C*) RNA derived from embryos. The age of the embryos in days is indicated above the lanes. (*D*) RNA derived from P19 cells treated (lane +) and not treated (lane −) with retinoic acid.

Assembly of the sequences derived from pPCR779 and the cDNA sequences generated a continuous sequence (see Fig. 1*C*) from which a putative translation product could be derived. Fig. 4 shows the first 1500 bases of the assembled Wnt-3 cDNA sequence as well as the predicted amino acid sequence of the Wnt-3 protein. The translation start codon is followed by an open reading frame of 1065 bases coding for 355 amino acids and ending in two adjacent stop codons (Fig. 4). This primary translation product has a calculated molecular mass of 39,660 daltons. The Wnt-3 translation product starts with a putative signal peptide, is very cysteine-rich, and contains two possible N-linked glycosylation sites. This putative Wnt-3 protein sequence is similar to the Wnt-1 (17, 18), Wnt-2 (19), and wingless (3) proteins, collectively called the Wnt family of proteins. Fig. 5 shows a lineup of the amino acid sequences of the members of the Wnt family of proteins.

Exon/intron boundaries of the *Wnt-3* gene were determined by use of oligonucleotide primers to generate sequences from both genomic fragments and cDNA fragments. Sites of divergence between the cDNA and genomic sequence, marking intron/exon and exon/intron boundaries,

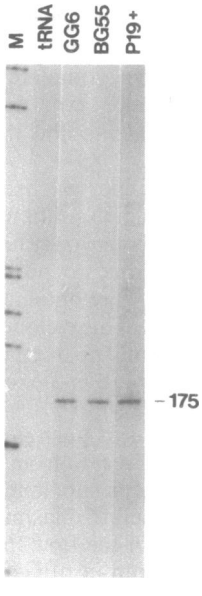

FIG. 3. RNase protection with RNA samples derived from tumors with an MMTV proviral integration in the *Wnt-3* locus, GG6 and BG55, as well as retinoic acid-treated P19 cells. As a probe a phage SP6 antisense transcript from pBG42 (lane tRNA) was hybridized to RNA samples at 55°C in the hybridization buffer as described in ref. 11. Lane M contains molecular mass standards.

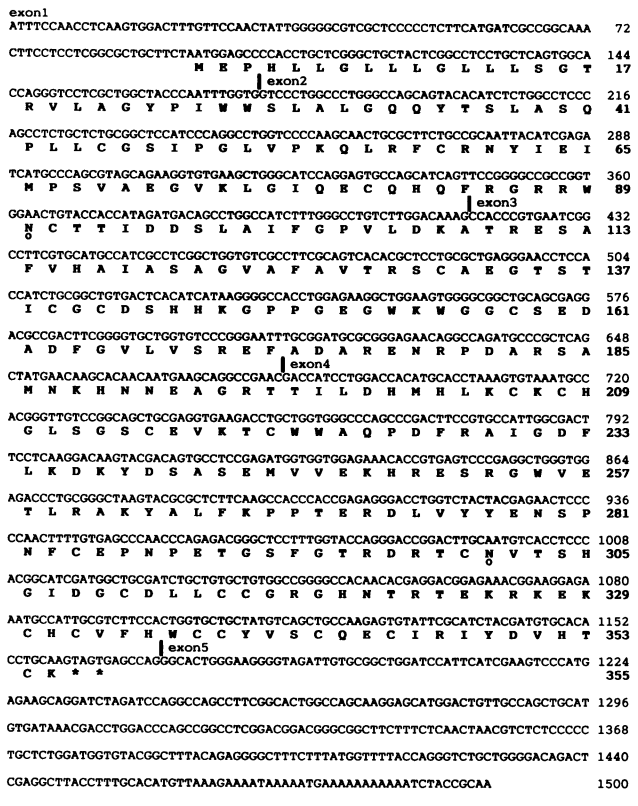

FIG. 4. Nucleotide sequence of the 5′ noncoding domain, the complete coding domain, and the first 340 bases of the 3′ noncoding domain representing the *Wnt-3* transcript. Bases 1–13 are derived from the sequence of the putative first exon, bases 14–352 are derived from the sequence of pPCR779, and bases 312–1500 are derived from the sequence of pCG3. The putative amino acid sequence in single-letter code is shown below the DNA sequence. Vertical lines indicate the positions of the exons in the represented primary transcript. The open circles indicate the positions of the possible N-linked glycosylation sites. Asterisks indicate termination codons.

were flanked in the genomic sequence by consensus splice acceptor and splice donor sites (20). The positions of the exons were established on the genomic map by linking sites present in each exon to the homologous sites in the genomic clones. All introns in the *Wnt-3* gene were at homologous positions compared with the *Wnt-1* gene, except for the intron between exons 4 and 5 in *Wnt-3*, which is unique. The fifth exon is completely noncoding.

Chromosome Mapping. We determined the chromosome on which *Wnt-3* is located by hybridizing probe pBG14 (Fig. 1) to a panel of mouse/Chinese hamster somatic cell hybrids (9). The *Wnt-3* hybridization pattern in the cell hybrids was concordant with genes and isoenzymes on chromosome 11. In addition, fine mapping of the *Wnt-3* area using interspecific backcross animals (21) between C57BL/6J and *Mus spretus* mice has located the gene 4.2 centimorgans distal from a cluster of genes containing c-*erb-A*, *Csfg*, and c-*erbB-2* and 4.9 centimorgans distal from the *hox-2* cluster (22).

DISCUSSION

The *Wnt-3* Locus, an Additional Protooncogene. We have cloned a common integration locus for MMTV on mouse chromosome 11 called *Wnt-3*. The *Wnt-3* area harbors five acquired MMTV proviruses in a large set, ≈100, of independent MMTV-induced mouse mammary tumors. The derived amino acid sequence of *Wnt-3* is strikingly similar to that of members of the *Wnt* family of genes. In the mouse, this family consists of *int-1*/*Wnt-1*, the *Wnt-1* related gene *irp*/*Wnt-2*

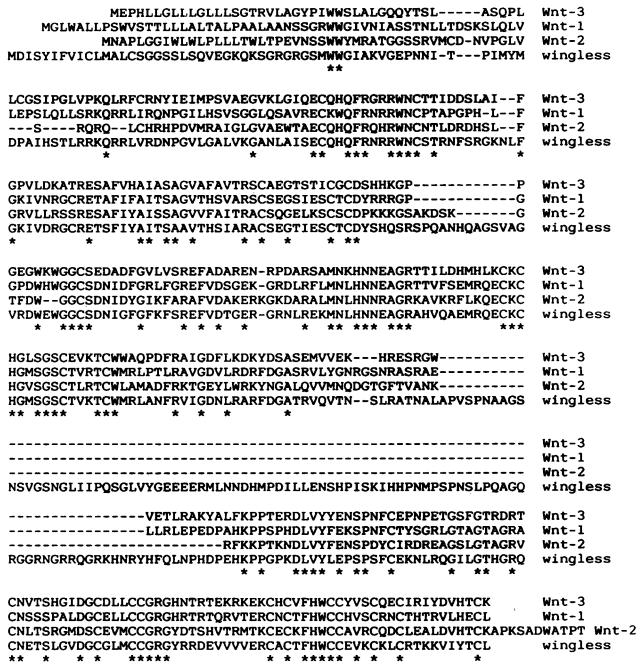

FIG. 5. Lineup of amino acid sequences in single-letter code of the mouse Wnt-3, Wnt-1, and Wnt-2 proteins and the *Drosophila melanogaster* wingless protein. Asterisks indicate sequence identity in all presented amino acid sequences.

(23), and *Wnt-3*. All of these genes are related to the *Drosophila* segment polarity gene wingless. The identity between *Wnt-1* and wingless genes is nevertheless higher than that of the others (Table 1). Identical amino acids in all four sequences are marked with an asterisk in Fig. 5. Besides the sequence homology between *Wnt-1* and *Wnt-3*, the structures of the genes are conserved as well. All introns, including the 36-kb first *Wnt-3* intron, are present at homologous positions, with the exception of the additional *Wnt-3* intron in the 3′ untranslated part. In all members of the *Wnt* family of genes, the first exon codes for the signal peptide; the subsequent first intron is always located between the first and the second guanosine of the codon for the second of two adjacent conserved tryptophan residues. Of the 24 cysteine residues present in the Wnt-3 protein, 22 are at identical positions. In addition one N-linked glycosylation signal is conserved in all members of the family, suggesting that this site is glycosylated and essential for the action of the mature proteins. *Wnt-1* and wingless genes encode secreted proteins associated with the extracellular matrix (24–26), and since the putative Wnt-3 protein also possesses a signal peptide, one can envisage that the Wnt-3 protein is exported from the cell. The capacity of both proteins to contribute to tumor formation in a tissue where these genes are normally not expressed suggests that they activate a Wnt-related ligand receptor present on mammary cells. The presence of such a receptor on mouse mammary epithelial cells also would suggest that a member of the Wnt family of proteins is the normal ligand for this receptor and may play a role in the normal growth of the mammary gland.

Table 1. Percentages of mutual identical/similar amino acid residues at homologous positions in the aligned protein sequences of the Wnt family

	Wnt-1	Wnt-2	wingless
Wnt-3	47/65	44/65	41/58
Wnt-1		42/63	57/71
Wnt-2			44/62

The normal expression of *Wnt-3* is very restricted—only seen in adult brain and in embryos. We detected the first sign of *Wnt-3* expression at day 10.5 in embryogenesis, while maximum expression is reached at day 12.5. The gene may be expressed earlier at lower levels, as very few (2 of 5×10^6) clones were isolated from a day 8.5 mouse embryo library. After day 12.5, *Wnt-3* expression declines to a lower level but remains detectable. In adult mice *Wnt-3* is expressed in the brain only. *Wnt-3* expression in P19 cells is concomitant with neural differentiation; the time course of *Wnt-3* expression correlates with the appearance of neural cell types in the cultures and is rather similar to expression of the *Wnt-1* gene (27).

Together, the data on the sites of *Wnt-3* expression suggest that the gene has, like *Wnt-1* (28), a function in the development of the nervous system, although the precise localization of the pattern of expression has to await *in situ* hybridization experiments.

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