MOLECULAR AND CELLULAR BIOLOGY, Aug. 1990, p. 4170-4179 Vol. 10, No. 8
0270-7306/90/084170-10$02.00/0
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The *Wnt-1* (*int-1*) Oncogene Promoter and Its Mechanism of Activation by Insertion of Proviral DNA of the Mouse Mammary Tumor Virus

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Received 27 November 1989/Accepted 7 May 1990

Wnt-1 (int-1) is a cellular oncogene often activated by insertion of proviral DNA of the mouse mammary tumor virus. We have mapped the 5′ end and the promoter area of the Wnt-1 gene by nuclease protection and primer extension assays. In differentiating P19 embryonal carcinoma cells, in which Wnt-1 is naturally expressed, two start sites of transcription were found, one preceded by two TATA boxes and one preceded by several GC boxes. In P19 cells, a 1-kilobase upstream sequence of Wnt-1 was able to confer differentiation-specific expression on a heterologous gene. We have investigated how Wnt-1 transcription was affected by mouse mammary tumor virus proviral integrations in various configurations near the promoters of the gene. One provirus has been inserted in the 5′ nontranslated part of Wnt-1, in the same transcriptional orientation, and has functionally replaced the Wnt-1 promoters. Wnt-1 transcription in this tumor starts in the right long terminal repeat of the provirus, with considerable readthrough transcription from the left long terminal repeat. Another provirus has been inserted in the orientation opposite that of Wnt-1 into a GC box, disrupting the first Wnt-1 transcription start site but not the downstream start site. Most insertions have not structurally altered the Wnt-1 transcripts and have enhanced the activity of the normal two promoters.

Insertion of proviral DNA of retroviruses can influence host cell gene expression in various ways, ranging from transcriptional activation or inactivation of genes to the production of mutated cellular proteins (reviewed in reference 38). Many cellular oncogenes have been identified originally as loci that are mutated in tumors as a consequence of proviral insertion (22, 38). The *int-1* gene, recently renamed *Wnt-1*, was the first example of such a gene, being frequently activated by insertion of proviral DNA of the mouse mammary tumor virus (MMTV) in carcinomas in the mammary gland (24). The oncogenic potential of *Wnt-1* has now been firmly established: the gene is able to transform cells in vitro (2, 26) and transgenic mice bearing *Wnt-1* linked to the MMTV long terminal repeat (LTR) develop mammary hyperplasia and tumors (35).

Wnt-1 has a normal function in early embryogenesis. The *Drosophila* homolog of *Wnt-1* was shown to be identical to the segment polarity *wingless* gene, a gene involved in pattern formation within individual segments (25). The *wingless* gene is part of a regulatory network regulating the number and differentiation of the segments and has been shown to be under the control of several other segmentation genes, including homeobox-containing genes (reviewed in reference 12). In mice, *Wnt-1* is expressed in a very restricted pattern in the developing neural system, in particular in specific locations in the neural plate and the folding neural tube (4, 41). In adult mice, there is expression of *Wnt-1* in the mature testis (32) but all other organs tested, including the normal mammary gland, lack detectable expression. In line with the expression of *Wnt-1* in the developing neural system is the observation that P19 embryonal carcinoma cells, which differentiate into neuroectodermal cells when treated with retinoic acid, switch on *Wnt-1* expression (30, 33). Most other embryonal carcinoma cells do not express the gene (14).

The nucleotide sequences of genomic *Wnt-1* DNA and of a cDNA clone have been established (6, 27, 37). The gene consists of four exons, transcribed into a 2.6-kilobase (kb) mRNA and translated into a cysteine-rich protein of 44 kilodaltons containing a signal peptide. The inserted proviruses in tumors are at various distances from the gene, sometimes within exons but always outside the protein-encoding domain, illustrating the dominant nature of the insertional mutations (37). Characteristically, the proviruses are oriented away from the *Wnt-1* gene itself, with a few exceptions (23). This orientation and the distance of some proviruses from the gene suggested that the transcriptional activation of *Wnt-1* in tumors is caused by enhancer elements in the MMTV provirus, working at a distance on the *Wnt-1* promoter (23). The typical orientation of the proviruses is probably required to avoid the interposition of the viral LTR promoter between the viral enhancer and the *Wnt-1* promoter; some enhancers have been shown to act only on the most proximal promoter (39). The structures of the *Wnt-1* promoter and of the 5′ end of the gene have not been established, however.

To learn more about the mechanism of activation of *Wnt-1* in tumors and to study the control of *Wnt-1* expression during normal development, we have determined the structure of the promoter of the gene by primer extension methods and nuclease protection experiments. We compared the structure of *Wnt-1* transcripts in tumors and in P19

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cells normally expressing the gene, and we defined an upstream *Wnt-1* sequence that confers differentiation-specific expression on a heterologous gene in P19 cells.

MATERIALS AND METHODS

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 SW27.1 rotor at 20,000 rpm. The pellet was dissolved in TE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA)–0.1% sodium dodecyl sulfate and extracted twice with phenol-chloroform (1:1). Total cellular RNA was stored as an ethanol precipitate at −20°C.

RNase protection experiments. For the detection of *Wnt-1* transcripts, an SP6 construct was made in which the 5′ end of the *Wnt-1* gene (a 2.3-kb fragment) was cloned into the antisense orientation in the vector SP6. To produce runoff transcripts, the plasmid was linearized with *Eco*RI. Runoff transcripts were synthesized in 20 μl of a reaction mixture consisting of 40 mM Tris hydrochloride (pH 7.5); 6 mM $MgCl_2$; 2 mM spermidine; 10 mM dithiothreitol; 30 U of RNasin (Boehringer); 500 μM (each) CTP, GTP, and ATP; 12.5 μM UTP; 25 μM ^{32}P-UTP (800 Ci/mmol, SP6 grade, Amersham Corp.); bovine serum albumin (0.1 μg/μl); linearized plasmid (±0.6 μg); and 10 U of SP6 polymerase (Boehringer) at 40°C for 1 h.

Solution hybridization and RNase mapping were essentially performed as described by Melton et al. (20). Total cellular RNA (20 μg) was hybridized overnight at 65°C in 30 μl of buffer containing 80% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.7), 400 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate to 10 ng of ^{32}P-labeled antisense SP6 RNA probe (3×10^6 dpm). Following hybridization, 300 μl of 0.3 M NaCl–10 mM Tris hydrochloride (pH 7.5)–5 mM EDTA–RNase A (20 μg per ml)–RNase T1 (3 U) was added and incubated for 30 min at 37°C. The RNase digestion was terminated by the addition of 20 μl of 10% sodium dodecyl sulfate and 50 μg of proteinase K and incubation for 15 min at 37°C. Protected RNA was extracted with phenol-chloroform (1:1) and precipitated with ethanol. Protected fragments were analyzed by denaturing acrylamide electrophoresis.

Primer extensions. For primer extensions, we essentially followed the protocol of Westaway et al. (40). Thirty-microgram samples of total RNA were precipitated with sodium acetate and ethanol and washed with 80% ethanol. Two independent primers were used for these experiments (see Fig. 2). RNA was dissolved in 10 μl of a solution containing 10 mM Tris hydrochloride (pH 8.3), 300 mM NaCl, 1 mM EDTA, and 0.25 pmol of kinase-labeled oligonucleotide. The oligonucleotides were kinase labeled to specific activities of more than 10^7 cpm/pmol. Hybridization was performed at 65°C for 60 min. The tubes were then transferred to 42°C, and after 5 min, their contents were diluted by the addition of 24 μl of a prewarmed solution containing 3.3 μl of 10× primer extension cocktail (600 mM NaCl, 100 mM Tris hydrochloride [pH 8.3], 0.5 mg of actinomycin D per ml, 80 mM $MgCl_2$), 3.3 μl of 10 mM dNTPs, 0.3 μl of dithiothreitol, and 17 μl of double-distilled water. Twenty units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) was added, the reaction mixtures were transferred after 30 min to 46°C to melt possible GC-rich sequences, and a second sample of reverse transcriptase (20 U) was added. The reaction was stopped after 30 min by the addition of a 1/10 volume of 3 M sodium acetate and two phenol-chloroform extractions followed by one chloroform extraction. Nucleic acids were precipitated with 2 volumes of ethanol, washed with 80% ethanol, dried, and suspended in 4 μl of a solution containing 80% deionized formamide, 1 mM EDTA, 10 mM NaOH, 0.1% bromophenol blue, and 0.1% xylene cyanol; solutions were heated at 100°C for 10 min and loaded directly onto a 0.375-mm-thick, 60-cm-long 6% sequencing gel. After the unhybridized input oligonucleotide primer had gone off the bottom of the gel, electrophoresis was terminated; the gel was then fixed with 10% methanol–10% glacial acetic acid, dried, and then exposed.

Nuclease S1 analysis of RNA. The 5′ end labeling was carried out by the method of Maxam and Gilbert (19). Typically, 5 μg of plasmid DNA was cut with a restriction enzyme, phenol extracted, and ethanol precipitated. Recovered DNA was dissolved in 450 μl of 50 mM Tris hydrochloride (pH 8.0), and 1 μl of a suspension containing 13 U of calf intestine alkaline phosphatase (Boehringer) was added. Incubation for 1 h at 37°C was followed by incubation for 1 h at 65°C to inactivate the enzyme. The DNA was purified on a DEAE-cellulose column and ethanol precipitated. The phosphatased, purified DNA was then dissolved in 10 μl of TE. To label the ends, 10 μl (100 μCi) of [γ-^{32}P]ATP (5,000 Ci/mmol; Amersham), 2 μl of 10× kinase buffer (500 mM Tris hydrochloride [pH 7.6], 100 mM $MgCl_2$, 50 mM dithiothreitol) and 1 μl (4.5 U) of T4 polynucleotide kinase (Boehringer) were added. The mixture was incubated for 20 min at 37°C, and labeled DNA was separated from unincorporated [γ-^{32}P]ATP on a Sephadex G-50 column.

Nuclease S1 analysis with end-labeled DNA was carried out according to the method of Van Ooyen and Nusse (37). Plasmid DNA was end labeled, cut with a second restriction enzyme, and electrophoresed on a 1% agarose gel in Tris-acetate buffer (40 mM Tris-acetate [pH 7.8] plus 1 mM EDTA, containing 0.5 μg of ethidium bromide per ml). The band of interest was cut out of the gel, and the gel material was heated to 65°C, frozen in liquid nitrogen, extracted with chloroform, and ethanol precipitated. The probe was purified on a DEAE-cellulose column equilibrated with TNE (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 100 mM sodium chloride). After elution with TNE containing 1.5 M NaCl, 100 μg of yeast RNA and 2.5 volumes of ethanol were added. DNA probe and RNA (both ethanol precipitates) were mixed, centrifuged, dried, and dissolved in a mixture containing 80% formamide, 40 mM 1.4-piperazine diethanesulfonic acid buffer (pH 6.4), 400 mM NaCl, and 1 mM EDTA. After being heated for 10 min at 70°C to denature the double-stranded DNA, the nucleic acids were allowed to hybridize overnight in a volume of 5 to 10 μl at various temperatures (45 to 55°C). Hybrids were chilled on ice, and 100 μl of nuclease S1 buffer (0.03 M sodium acetate [pH 4.5], 0.25 M NaCl, 1 mM $ZnSO_4$) containing single-stranded salmon sperm DNA (20 μg/ml) and 40 U of nuclease S1 (P-L Biochemicals, Inc.) was added. After 45 min at 30°C, the mixture was phenol extracted and ethanol precipitated. Samples were dissolved in loading dye (60 mM Tris-borate [pH 8.3]–1 mM EDTA [TBE], 80% formamide, 0.1% (each) bromophenol blue and xylene cyanol) and applied to a 6.5% polyacrylamide gel in 60 mM TBE buffer containing 7 M urea.

DNA sequence analysis was carried out by the method of Maxam and Gilbert (19) on polyacrylamide gels in 50 mM TBE buffer containing 7 M urea.

Constructs, DNA transfections, and chloramphenicol ace-

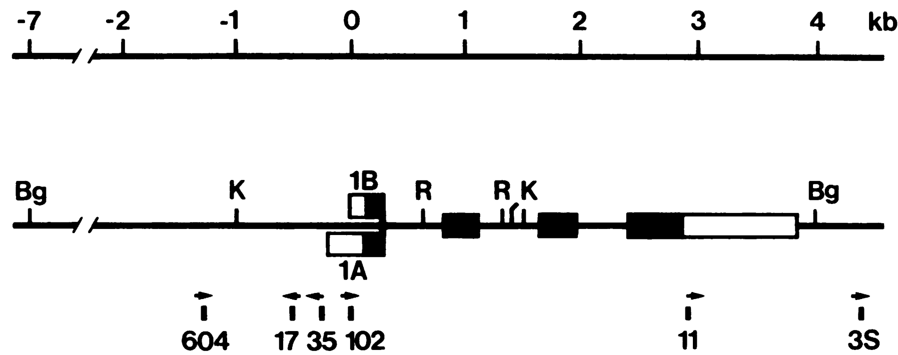

FIG. 1. Organization of the *Wnt-1* gene. The four coding exons of *Wnt-1* are shown as boxes, with the parts encoding the protein in black. Arrows indicate integration sites and orientations of MMTV proviruses in different tumors. R, *Eco*RI; Bg, *Bgl*II; K, *Kpn*I.

tyltransferase (CAT) assays. The *Wnt-1*-CAT plasmids used for transfection on P19 cells were constructed according to common molecular cloning techniques (18). DNA fragments derived from the *Wnt-1* upstream region were ligated into the polylinker of pUC12CAT, with use of the Klenow fragment of DNA polymerase and synthetic linkers, if necessary.

P19 cells were grown in a 1:1 mixture of Dulbecco modified Eagle medium and HamF12 with 5% fetal calf serum at 5% CO_2. To induce differentiation into the neural pathway, the cells were grown for 6 days as aggregates (or embryoid bodies) in bacterial-grade petri dishes in the presence or absence of 10^{-7} M retinoic acid. The embryoid bodies were subsequently transferred to tissue culture grade petri dishes and kept in culture for an additional 6 days in the presence of 10^{-7} M retinoic acid (5). For expression studies, P19 cells were grown as a monolayer in a 1:1 mixture of Dulbecco modified Eagle medium and HamF12 medium supplemented with 10% fetal bovine serum. Roughly 10^6 cells were transfected with 20 μg of cesium chloride-purified supercoiled plasmid DNA according to the protocol of Graham and Van der Eb (9). The precipitate was left on the cells overnight. The next day, cells were washed with medium or phosphate-buffered saline and refed. In the coselection experiments, transfected P19 cells were selected by culturing in 800 μg of G418 per ml. Colonies were pooled, and if required, retinoic acid was added in the medium at a concentration of 10^{-7} M and the mixture was incubated for 48 h. After approximately 48 h, cells were harvested and a protein extract was prepared by sonification or three repeated freeze-thaw cycles. Subsequently, 200 μg of total protein was used in the CAT assay that was performed essentially according to the procedure described by Gorman et al. (8).

Molecular cloning of viral DNA host cell DNA junction fragments. The fragments from tumors 102 and 604 were cloned as *Eco*RI fragments into bacteriophage gtWES, and the inserts in tumors 35 and 17 were cloned as *Sac*I fragments into the same vector (18).

Polymerase chain reaction. RNA isolated from tumor 102 was assayed for the presence of readthrough transcripts from the proviral DNA into *Wnt-1* by using the polymerase chain reaction (15). Total RNA was first transcribed into DNA with reverse transcriptase and random hexamer primers, and the DNA was amplified for 30 cycles with two sets of specific primers derived from the *Wnt-1* and the MMTV LTR sequence. Primer A (5′ TTTGTGTCTGTTCGCCATCC 3′) was was from the MMTV U3 sequence, primer B (5′ GTAAATGCTTATGTAAACC 3′) was from the U3 sequence, and primer C (5′ TTCTGACGACTGTGGTTGCT 3′) was from the *Wnt-1* sequence. The products were analyzed by agarose gel electrophoresis.

RESULTS

Nucleotide sequence of the *Wnt-1* promoter area and the 5′ end of the gene. Previously we had analyzed the structure of the transcriptional unit of *Wnt-1* by nuclease S1 protection experiments using RNA from mouse mammary tumors, combined with nucleotide sequence analysis of the genomic copy of the gene (37). By using various probes, it was established that *Wnt-1* consisted of four exons (Fig. 1). The 5′exon consisted of two forms (1A and 1B) with identical 3′ ends but different 5′ ends, which could be either starts of transcription or splice acceptor sites.

To determine the 5′ end of *Wnt-1*, we have established more of the *Wnt-1* upstream nucleotide sequence, performed additional nuclease protection experiments, and determined the start of transcription by primer extension assays. For these experiments, we used RNA from two sources. As in our previous study (37), we took RNA from a tumor (in this case, an established cell line from a mammary tumor, 3S) in which the gene is activated by insertion of a provirus. This particular provirus was mapped at the 3′ end of the gene (Fig. 1) and has presumably activated the *Wnt-1* promoter by enhancement without affecting the structure of the promoter itself (Fig. 1). To exclude the possibility that proviral activation of *Wnt-1* had nevertheless affected the structure of the transcript, we used the P19 cell line as a source of normal *Wnt-1* RNA. When P19 cells were induced to differentiate by the addition of retinoic acid, they started to express the *Wnt-1* gene to reasonably high levels, presumably from its natural promoter.

In Fig. 2, we show the extended upstream sequence of *Wnt-1*, with the positions of the primers used for the analy-

FIG. 2. Nucleotide sequence of *Wnt-1* promoter area. The start 2 site is at position +1. The positions of restriction enzyme cleavage sites used in this study are given below the sequence; integration sites of MMTV proviruses as mapped by sequence analysis are indicated by arrows. The two primers used for primer extension analysis are double underlined. TATA- and GC-rich boxes are shaded. ATTA sequences implicated in homeobox protein binding are underlined. The whole *Wnt-1* sequence is present in GenBank under accession number M34750.

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      ↓T604
TTTGCTAATCTTTCTTTAAAAAACTGTTTCAGGAGATTTATTTTATGTAT  -1601
               DraI
500 nucleotides

TCTAATGATAAGCACAGGTTGACTCAAGGTGCCATAGAGTGACACTAGGT  -1051
                                              KpnI
ACCCAGAGCGACAGAATGACACCTATGAGTGCACGTCGTTAATCACAAAC  -1001

ACACACACACACACACACACACACACACACACACACTCATGCACCCACCT   -951

GCAAACACAATTGCAGCCTTCTGGACGTCTCCTGTCACAGCCCCACCTCC   -901

TTCCTGATACACTGCGTTAAGTGGTGACTGTAACAAAATGACTTCATGCT   -851

CTCCCTGTCCTGAGCCAAATTACACAATTATTTGGAAAGGGCTCAAAATG   -801

TTCTTCGTTAGAAGTTTCTGGATACACCAATACACAGGAGCGTGCACCCT   -751

CAGAACACATGTACACTTTGACTTAATCTCACGGGTGACACACCGACGCT   -701

TACACTCCCCCTAGCCCACAGAGGCAAACTGCTGGGCGCTTCTGAGTTTC   -651

TCACTGCCACCAGCTCGGTTTGCTCAGCCTACCCCCGCACCCCGCGCCCG   -601
                                              SmaI
GGAATCCCTGACCACAGCTCCACCCATGCTCTGTCTCCTTCTTTTCCTTC   -551

TCTGTCCAGCCGTCGGGGTTCCTGGGTGAGGAAGTGTCTCCACGGAGTCG   -501
                            ↓T17
CTGGCTAGAACCACAACTTTCATCCTGCCATTCAGAATAGGGAAGAGAAG   -451

AGACCACAGCGTAGGGGGGACAGAGGAGACGGACTTCGAGAGGACAGCCC   -401

CACCGGCGCGTGTGGGGGAGGCAATCCAGGCTGCAAACAGGTTGTCCCCA   -351

GCGCATTGTCCCCGCGCCCCCTGGCGGATGCTGGTCCCCGACGGGCTCCG   -301

GACGCGCAGAAGAGTGAGGCCGGCGCGCGTGGGAGGCCATCCCAAGGGGA   -251
                   NaeI
GGGGTCGGCGGCCAGTGCAGACCTGGAGGCGGGGCCACCAGGCAGGGGGC   -201
↓T35                                 ──►start 1
GGGGGTGAGCCCCGACGGTTAGCCTGTCAGCTCTTTGCTCAGACCGGCAA   -151

GAGCCACAGCTTCGCTCGCCACTCATTGTCTGTGGCCCTGACCAGTGCGC   -101

CCTGGTGCTTTTAGTGCCGCCCGGGCCCGGAGGGGCAGCCTCTTCTCACT    -51
                  SmaI  primer2 ══════════════════════
GCAGTCAGCGCCGCAACTATAAGAGGCCTATAAGAGGCGGTGCCTCCCGC     -1
═════════════════════             StuI
──►start 2                              ↓T102
AGTGGCTGCTTCAGCCCAGCAGCCAGGACAGCGAACCATGCTGCCTGCGG     50

CCCGCCTCCAGACTTATTAGAGCCAGCCTGGGAACTCGCATCACTGCCCT    100
primer1 ══════════════════════════════════════════
CACCGCTGTGTCCAGTCCCACCGTCGCGGACAGCAACCACAGTCGTCAGA    150

ACCGCAGCACAGAACCAGCAAGGCCAGGCAGGCCatggggctctgggcgc    200
                        SfiI      NcoI
tgctgcccagctgggtttctactacgttgctactggcactgaccgctctg    250
         PvuII
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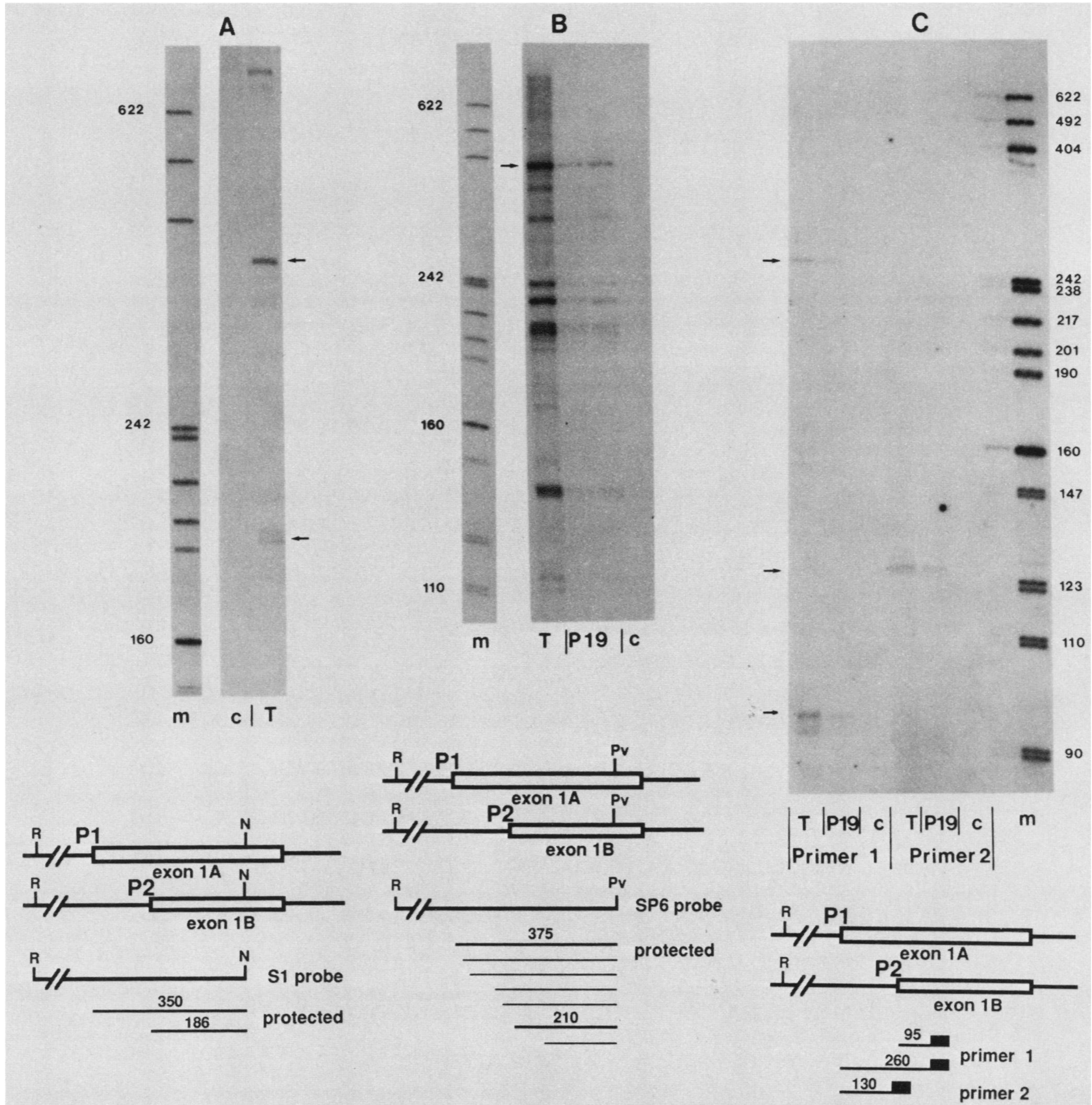

FIG. 3. Mapping the 5′ end of *Wnt-1*. Shown is a combination of the S1 nuclease protection (A), RNase protection (B), and primer extension (C) experiments. The positions of the probes and primers with respect to the emerged structure of the 5′ end are shown below the gels. Lines indicate the products obtained from the three types of analysis, with numbers indicating the sizes in nucleotides. Thin lines in panel B indicate the additional protected fragments seen with the RNase protection assay only. Lanes: m, marker (a *Hpa*II digest of PAT153 DNA labeled by Klenow DNA polymerase; sizes given in nucleotides); c, control (yeast tRNA); T, RNA from tumor cell line 3S; P19, RNA from retinoic acid-treated P19 cells. Arrows indicate the most prominent fragments, corresponding to the two major start sites which were obtained in all three types of experiments.

sis, the positions of various restriction enzyme sites used in this study, and the integration sites of MMTV proviruses in this area as found in various tumors (see below).

Figure 3 shows the combined results of the S1 nuclease (panel A), RNase protection (panel B), and primer extension assays (panel C). An S1 nuclease analysis with a 2,800-nucleotide probe labeled at the *Nco*I site, on RNA from the tumor cell line, confirmed the results obtained before: two major protected fragments, around 350 and 185 nucleotides, corresponded to two discontinuities representing the 5′ ends of exons 1A and 1B. The shorter protected fragment was resolved into a doublet. A third major protected fragment of

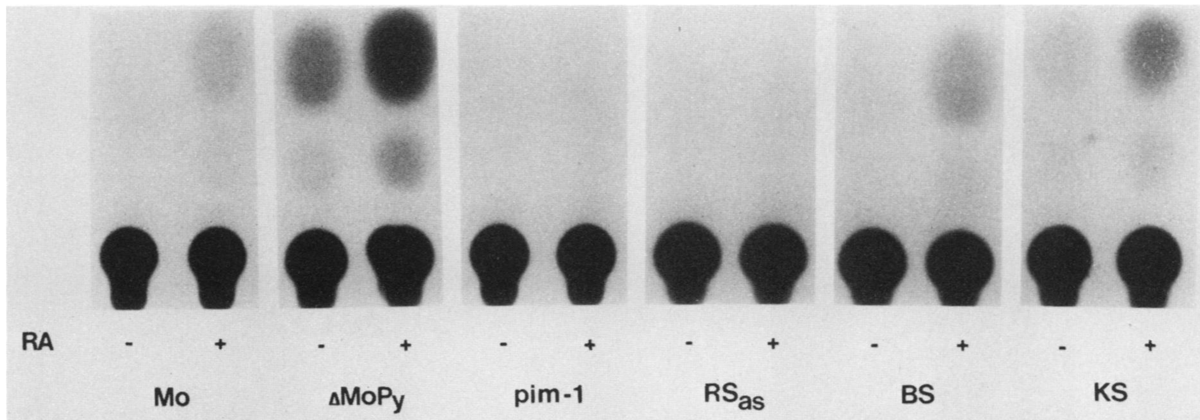

FIG. 4. Induction of *Wnt-1* expression by retinoic acid: CAT assays on P19 cells. Undifferentiated P19 cells were transfected with the constructs indicated, together with the G418-selectable marker pSV2neo. Colonies were pooled and either nontreated (−) or treated (+) with retinoic acid (RA). The lowest spots represent nonacetylated ^{14}C-chloramphenicol; upper spots represent acetylated (converted) forms of ^{14}C-chloramphenicol. In the constructs indicated, the following promoter elements were used to drive the CAT reporter gene: Mo, Moloney MuLV LTR; ΔMoPy, Moloney MuLV LTR enhancer replaced by the polyomavirus enhancer; pim-1, the *pim-1* promoter in combination with a Moloney MuLV enhancer; RS_{as}, a 1.5-kb *Eco*RI-*Sfi*I *Wnt-1* upstream promoter fragment placed in antisense orientation, serving as a negative control; BS, a 7-kb *Bgl*II-*Sfi*I *Wnt-1* upstream promoter fragment; KS, a 1.2-kb *Kpn*I-*Sfi*I *Wnt-1* upstream promoter fragment. See text for details of and references for constructs.

approximately 700 nucleotides had not been detected before; since it was not observed in the subsequent RNA mapping experiments, we did not further analyze the origin of this fragment. Additional minor S1 protected fragments between the two major fragments were found.

With the RNase protection experiments (Fig. 3B) with an SP6-generated probe and RNA from tumor cells and from P19 cells, the results were similar: major protected fragments corresponded to the sizes of the two overlapping exons (Fig. 3B, arrows), but the abundance of the other fragments was higher than in the S1 analysis. A major protected fragment of approximately 140 nucleotides was uniquely found in the RNase protections and not in the S1 mapping experiments. The reason for these differences was not clear. Varying the hybridization or RNase digestion conditions according to the methods of Myers et al. (21) did not significantly change the result. The P19 and the tumor cell RNAs yielded the same fragments.

The primer extension assays showed that the major discontinuities in the RNA protection experiments represented starts of transcription rather than splice sites. Primer 1 gave fragments of 95 and 260 nucleotides, corresponding to its distance from the 5′ ends of exons 1A and 1B; primer 2 gave one fragment of 130 nucleotides, the distance to the 5′ end of exon 1A. The extended product of 95 nucleotides was, like the S1 and SP6 product, split into two bands.

The combined outcome of these assays is projected on the nucleotide sequence of the *Wnt-1* promoter in Fig. 2. It appears that *Wnt-1* has two major start sites of transcription: the shortest form of the RNA (exon 1B, start 2) arbitrarily set at position 1, with another start (start 1, exon 1A) at position −160. Inspection of the nucleotide sequence showed the presence of two TATA boxes upstream of start 2, at −35 and at −25. Upstream of exon 1A, no TATA boxes were found, but the region was very GC rich and contained at least two SP1-binding sites.

Functional analysis of the *Wnt-1* promoter. Having mapped the start sites of *Wnt-1* transcription, we next analyzed whether the upstream area could function as a cell type-specific transcriptional promoter. For this purpose, a number of constructs were made in which different *Wnt-1* promoter fragments served to direct the expression of the bacterial reporter gene CAT. In transient assays on differentiated P19 cells, no CAT activity was measured, which we attributed to a low efficiency of transfection. We then cotransfected undifferentiated P19 cells with the *Wnt-1*-CAT constructs and *neo* as a selectable markers selected for G418 resistance, pooled colonies, and tested these for CAT activity before and after retinoic acid treatment (Fig. 4).

A low but measurable basal level of CAT activity as well as a considerable increase after retinoic acid treatment was observed with the *Wnt-1*-CAT construct KS. This plasmid contained approximately 1 kb of *Wnt-1* promoter sequence, extending from the *Sfi*I site at position 175 (between start 2 and initiator ATG) (Fig. 2) to the *Kpn*I site at position −1050. The expression levels found with a longer construct, BS, which contained about 7 kb of *Wnt-1* promoter sequence extending from the *Sfi*I site (position 175) to a *Bgl*II site at position −7 kb, were somewhat lower. As a negative control, a construct with a 1.5-kb *Eco*RI-*Sfi*I (*Sfi*I at position −175) *Wnt-1* promoter fragment placed in inverted orientation towards the CAT gene (RS_{as}) showed no expression. The activity of the *Wnt-1* constructs was specific for P19 cells: expression of the *Wnt-1* promoter-CAT constructs in transient assays in several other cell types (NIH 3T3, HeLa, MCF7, T47D, XC, and GRSL T-cell leukemia) was negative, regardless of the presence of retinoic acid, whereas positive controls worked as expected (data not shown).

We measured the CAT activity of several other constructs in stably transfected P19 cells to check the transcriptional activity of known other promoters and to correlate promoter activity to the state of differentiation of the cells (Fig. 4). Transcription of many retroviruses is blocked in undifferentiated EC cells, and this block can be relieved upon differentiation (7). *Mo*, a construct in which the CAT gene is driven by the Moloney murine leukemia virus (MuLV) LTR and which is inactive in undifferentiated F9 EC cells (17), was also not expressed in noninduced P19 cells. δMoPy, in which the Moloney LTR enhancer is replaced by an enhancer from a mutant polyomavirus to make it active in undifferentiated F9 EC cells (17), was also expressed in untreated P19 cells. Both of these constructs showed in-

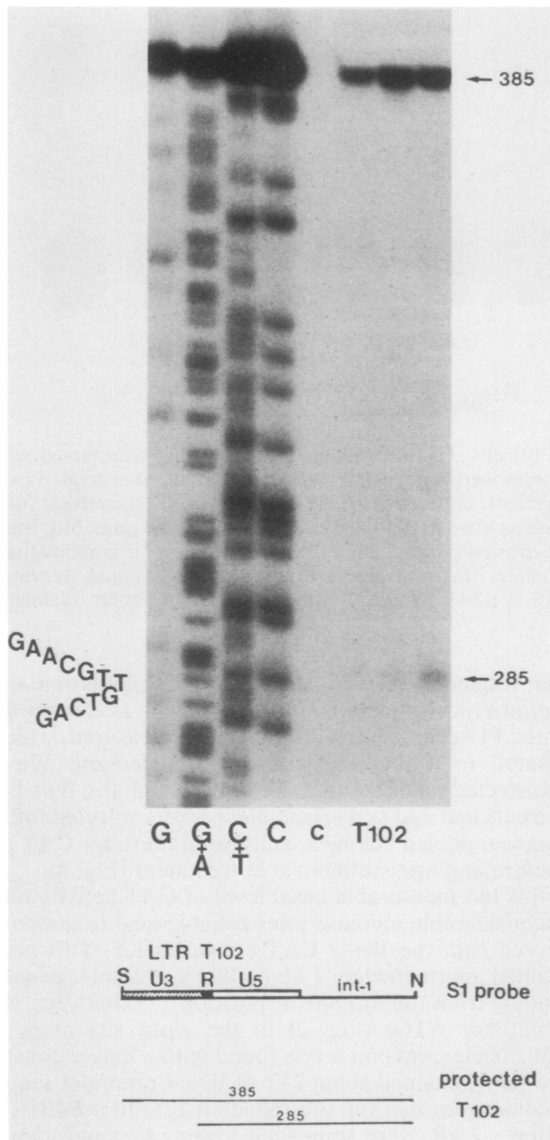

FIG. 5. S1 nuclease mapping of *Wnt-1* transcription in tumor 102. The S1 probe, shown below the gel, was a *Sac*I-*Nco*I fragment derived from the cloned junction fragment and contained part of the *Wnt-1* gene and a part from the MMTV LTR with the U3, R, and U5 domains. The probe was labeled at the *Nco*I site, and fragments of 385 and 284 nucleotides were protected (indicated by arrows). S1-treated material was run along a Maxam-Gilbert sequence ladder of the same fragment. c, Control tRNA; T102, tumor 102 RNA.

creased CAT activity upon differentiation. However, a *pim-1*-CAT construct which is active in lymphoid cells (16) was not active in untreated or differentiated P19 cells. In this plasmid, the CAT gene is driven by the *pim-1* promoter supported by the Moloney MuLV enhancer.

Influence of MMTV insertion on *Wnt-1* transcripts: the right LTR of the MMTV provirus in tumor 102 functionally replaces the *Wnt-1* promoter. In Fig. 2, in which we show the structure and sequence of the *Wnt-1* promoter area, we also indicate the insertion sites of several MMTV proviruses which map close to the promoter. All of these insertion sites have been determined by molecular cloning of proviral-cellular DNA junction fragments and nucleotide sequence analysis across the insertion site (37). The proviruses in

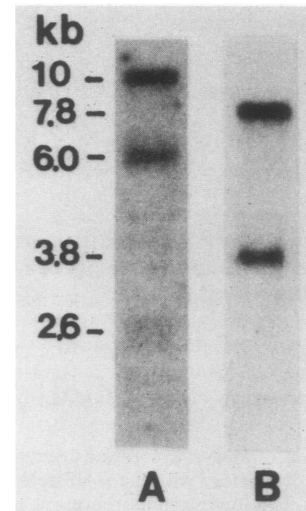

FIG. 6. RNA blot analysis of tumor 102 RNA, hybridized with a *Wnt-1* probe (lane A) and subsequently on the same filter with an MMTV LTR probe (lane B). Sizes of hybridizing RNA species were calculated from rRNA markers or known from the structure of the MMTV genome.

tumors 604 and 102 are inserted in the same transcriptional orientation as *Wnt-1*, whereas proviruses in tumors 17 and 35 are inserted in the opposite orientation. From restriction enzyme mapping on tumor DNA, it appeared that none of these proviruses had undergone rearrangements or deletions.

Some of these insertions were close to the *Wnt-1* start sites, in particular in tumor 102, in which the provirus had replaced the *Wnt-1* promoters. We have analyzed how this insertion has affected the structure of the resulting *Wnt-1* mRNA by nuclease S1 mapping of the transcripts. To this end, we used several S1 probes, one of which was derived from the cloned proviral DNA-cellular DNA junction fragment from tumor 102 (Fig. 5). This probe was labeled at the *Nco*I site in *Wnt-1* and hybridized to RNA from tumor 102. Two protected fragments were obtained, one of 385 nucleotides, the length of the probe, and one of 285 nucleotides. By comparison with a sequence ladder of the same probe, it appeared that the 285-nucleotide fragment mapped at the boundary between the U3 and R regions in the right MMTV LTR. Thus, *Wnt-1* transcription in this tumor was indeed caused by a promoter insertion and starts in the right MMTV LTR at the normal viral cap site.

The abundance of the 385-nucleotide protected fragment indicated that considerable amounts of transcripts were coming from more upstream promoters. By examining tumor 102 RNAs by their sizes on Northern (RNA) blots, we found that there were indeed long RNAs which were probably readthrough transcripts from the left LTR. With *Wnt-1* as a probe on the blot, three RNAs of 10, 6 and 2.6 kb were detected (Fig. 6). When the same blot was probed with an MMTV LTR, the two usual MMTV transcripts of 7.8 and 3.8 kb were found. It thus appeared that the two large *Wnt-1* transcripts had sizes corresponding to those of the two MMTV messengers, for *gag-pol* and *env* gene expression, plus those of the *Wnt-1* normal transcripts and had most likely arisen by readthrough over the right MMTV LTR into the *Wnt-1* gene. The shorter *Wnt-1* transcript, of 2.6 kb, is then the product of the start in the right LTR and presumably the only functional mRNA, i.e., giving rise to the *Wnt-1* protein.

Further evidence for the hybrid nature of these transcripts was obtained by applying the polymerase chain reaction (15) to RNA from tumor 102. The primers were derived from the *Wnt-1* (primer C) and the MMTV LTR U5 (primer A) and U3 (primer B) sequences (see Materials and Methods). Primer combination A-C gave a product of approximately 215 nucleotides, and combination B-C gave a product of 310 nucleotides, showing that the tumors indeed contained transcripts starting upstream from the cap site in the right MMTV LTR and proceeding into the *Wnt-1* sequence (data not shown). The readthrough mRNAs could not be detected on the blot hybridized to the MMTV probe because of the overabundance of the MMTV mRNAs that came not only from the provirus next to *Wnt-1* but also from many other proviruses present in this tumor.

Other insertions upstream from the *Wnt-1* promoter. Using different probes and RNA from the tumors 35, 17, and 604, in which MMTV insertions map close to the *Wnt-1* promoter and various S1 probes, we found the two major start sites mostly unaffected. In Fig. 7, we show an analysis with the junction fragment from tumor 35 as a probe, labeled at the *NcoI* site in *Wnt-1*. In tumor 11, a control sample from a tumor with a downstream provirus (Fig. 1), the probe revealed the two major start sites, at 350 and 186 nucleotides from the *NcoI* site. Tumor 604 has an MMTV insertion upstream but in the same transcriptional orientation as *Wnt-1*. The same two protected fragments in addition to a longer fragment of 385 nucleotides, were observed. The latter fragment had a size corresponding to the distance from the *NcoI* site in *Wnt-1* to the particular provirus in tumor 35 and is therefore presumably generated from a longer RNA, but is continuous with the probe only over the 385 nucleotides. Blot analysis of tumor 604 RNA did indeed reveal long transcripts, but there was not sufficient resolution for a more detailed analysis. Most likely, tumor 604 does contain readthrough transcripts from the provirus into *Wnt-1*, but the start sites of transcription are normally used.

An exception was tumor 35, with the MMTV insert in one of the GC boxes. The shorter form of *Wnt-1* RNA started normally at the initiation site downstream from the TATA boxes, yielding the 186-nucleotide fragment, but the first start was replaced. An S1 protected fragment of 400 nucleotides was found, indicating that the start of transcription is located in the MMTV LTR. Tumor 17 had contained *Wnt-1* transcripts starting at the normal sites (data not shown).

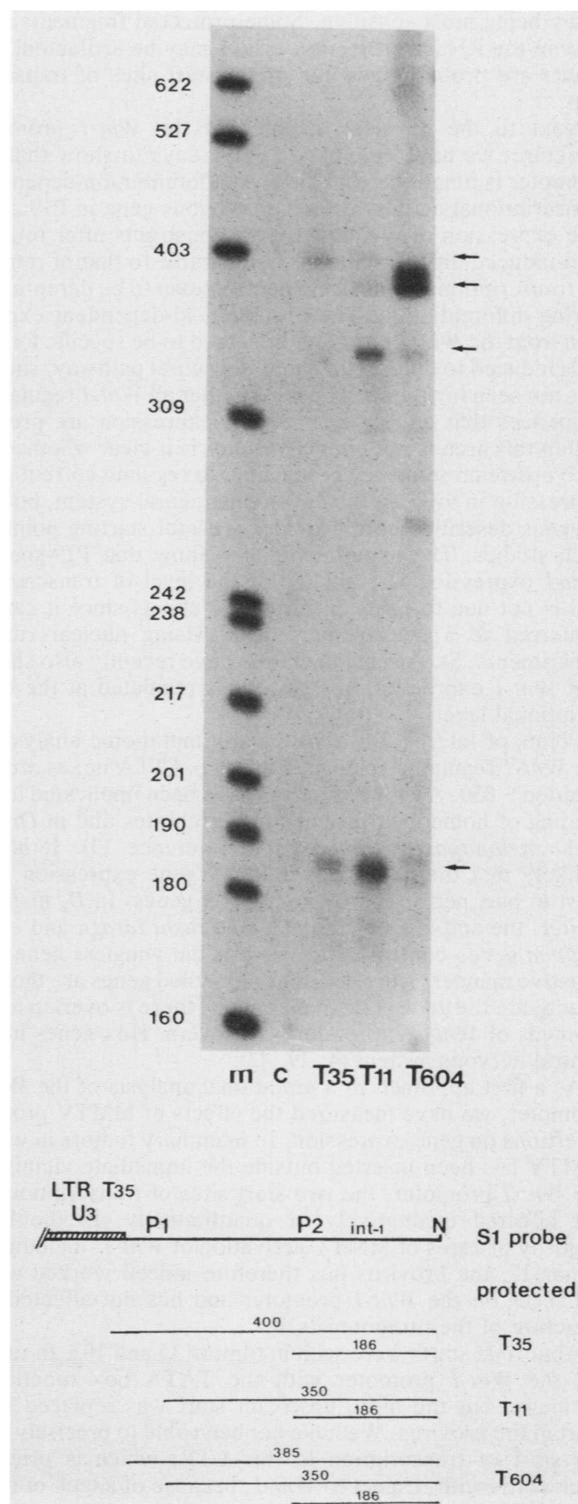

FIG. 7. S1 nuclease analysis of *Wnt-1* RNA in tumors 35, 11, and 604. The probe was a fragment derived from the cloned junction fragment from tumor 35, where the provirus was inserted backwards to the *Wnt-1* promoters P1 and P2, labeled at the *NcoI* site. Sizes of protected fragments (arrows in upper panel) in different tumors are indicated by thin lines below the gel and are given in nucleotides. m, Marker lane with pBR322 digested by *HpaII* and labeled by kinase; c, control tRNA.

DISCUSSION

From the analysis of the structure of the *Wnt-1* transcripts presented here, it appears that the gene has two major initiation sites of transcription, each one containing a different promoter element. Promoter 1, giving rise to exon 1A, contains a GC-rich area, with several SP1-binding sites. GC boxes are usually found in multiple copies, and they have been shown to cooperate in regulating the initiation of transcription (1). The GC boxes at *Wnt-1* are all conserved between the human and mouse *Wnt-1* genes (36), and the insertional mutation in tumor 35 disrupts the activity of this promoter, making it likely that these elements are indeed functioning in promoter activity. A second *Wnt-1* start was mapped at position +1. This promoter contains two closely spaced TATA boxes, probably directing the double start of exon 1B as found in all RNA mapping experiments.

In between the fragments corresponding to the two major starts, we found several other minor fragments. These were observed with all three techniques, but the relative intensi-

ties of the bands varied significantly, the RNase protection assay being most sensitive. Some protected fragments seen only in the RNase protection assays may be artifactual, but others are probably genuine minor start sites of transcription.

Next to the physical mapping of the *Wnt-1* promoter structure, we have employed CAT assays to show that the promoter is functional and confers differentiation-dependent transcriptional activity on a heterologous gene in P19 cells. The expression of the *Wnt-1*-CAT constructs after retinoic acid-induced differentiation is comparable to that of retroviral transcriptional control elements known to be derepressed during differentiation. The retinoic acid-dependent expression from the *Wnt-1* promoter appeared to be specific for P19 cells induced to differentiate into the neural pathway, since it was not seen in other cell lines. Whether all *Wnt-1* regulatory sequences that govern cell-specific expression are present within this area is not known yet, nor is it clear whether the 1-kb upstream sequence is sufficient to regulate correct gene expression in vivo, in the developing neural system, but the analysis described here provides a useful starting point for such studies. The experiments also show that P19-specific *Wnt-1* expression is regulated at the level of transcription and is not due to posttranscriptional effects since it can be conferred to a heterologous gene. Using nuclear run-on experiments, St. Arnaud et al. (33) have recently also shown that *Wnt-1* expression in P19 cells is regulated at the transcriptional level.

Points of interest for a systematic mutational analysis of the *Wnt-1* regulatory elements are two ATTA boxes around position −830. ATTA sequences have been implicated in the binding of homeobox proteins in vertebrates and in *Drosophila melanogaster* (reviewed in reference 13). It is not unlikely that the control over *Wnt-1* gene expression is at least in part performed by homeobox genes. In *D. melanogaster*, the activities of the pair-rule *fushi tarazu* and *even-skipped* genes control expression of the wingless gene in a negative manner, whereas other pair-ruled genes are thought to activate the gene (13). In mammals, there is overlap in the domains of *Wnt-1* expression and several Hox genes in the central nervous system (4, 29, 42).

As a first approach to a mutational analysis of the *Wnt-1* promoter, we have measured the effects of MMTV proviral insertions on gene expression. In mammary tumors in which MMTV has been inserted outside the immediate vicinity of the *Wnt-1* promoter, the two start sites of transcription are not affected qualitatively or quantitatively. In the large majority of cases of MMTV activation of *Wnt-1*, including in tumor 17, the provirus has therefore indeed worked as an enhancer on the *Wnt-1* promoter and has not affected the structure of the promoter itself.

Abnormal starts were seen in tumors 35 and 102. In tumor 35, the *Wnt-1* promoter with the TATA box functioned normally, but the more upstream start was replaced by a start in the provirus. We have not been able to precisely map the start of transcription in this LTR, which is oriented backwards with respect to *Wnt-1*, because of a lack of more material.

Tumor 102 is the only clear example of a promoter insertion at *Wnt-1*, a mechanism more commonly observed in other cases of insertional activation of host cell oncogenes. The S1 experiments with the junction fragment from tumor 102 as a probe showed that the right proviral LTR served as a functional promoter, leading to transcripts starting at the boundary between U3 and R. The level of RNA from the right LTR was, however, much lower than the amount of readthrough RNA presumably coming from the left LTR, as also seen in the RNA blot analysis of the *Wnt-1* transcripts. Such promoter occlusion phenomena have been observed before at other retroviruses, in which the left LTR is usually more active than the right LTR, and have been invoked to explain the frequent deletions in proviruses at the c-*myc* locus (3, 28). c-*myc* is usually activated by insertions of avian leukosis virus in such a way that the viral LTR replaces the normal c-*myc* promoter. In a study devoted to starts in the right LTR of complete avian leukosis virus proviruses by Herman and Coffin (10), their occurrence was estimated to be much lower than was observed for tumor 102. This was measured in nonselected integrations, whereas the insert of MMTV near *Wnt-1* and its resulting expression has been selected for during tumorigenesis.

Remarkable in tumor 102 are the readthrough *Wnt-1* transcripts, starting in the left LTR and proceeding over the right LTR into adjacent host cell sequences. Even the two differently spliced versions of MMTV RNA are both extended into *Wnt-1* sequences. The level of readthrough transcription is in the same range (15% of viral RNA) as that estimated by Herman and Coffin (10) for avian leukosis virus proviruses. It would seem that in this tumor, all conditions necessary to transduce *Wnt-1* into the MMTV viral genome are fulfilled (11); such readthrough RNAs are thought to be the first step in packaging oncogene RNA into the retroviral particle, followed by recombination in the infected target cell (34). MMTV variants with host cell-derived oncogenes have, however, never been found. The incidence of promoter insertion activation of the *int* genes is perhaps too low to allow such events to occur. Out of some 50 cases of *Wnt-1* activations seen in our laboratory, only one other example of an insert upstream of the gene in the same orientation has been found, in tumor 604. Although readthrough transcription presumably coming from the provirus was seen, the normal start sites of *Wnt-1* expression were used, indicating that this insertion has acted as an enhancer on the *Wnt-1* promoter rather than as a promoter insertion.

ACKNOWLEDGMENTS

We thank Liesbeth van Deemter for assistance in cell culturing and Ronald Plasterk, Piet Borst, Anton Berns, and Jacky Russell for comments on the manuscript.

These studies were supported by the Netherlands Cancer Foundation.

LITERATURE CITED

1. **Barrera-Saldana, H., K. Takahashi, M. Vigneron, A. Wildeman, I. Davidson, and P. Chambon.** 1985. All six GC-motifs of the SV40 early upstream element contribute to promoter activity in vivo and in vitro. EMBO J. **4:**3839–3849.
2. **Brown, A. M. C., R. S. Wildin, T. J. Prendergast, and H. E. Varmus.** 1986. A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. Cell **46:**1001–1009.
3. **Cullen, B. R., P. T. Lomedico, and G. Ju.** 1984. Transcriptional interference in avian retroviruses—implications for the promoter insertion model of leukemogenesis. Nature (London) **307:**241–245.
4. **Davis, C. A., and A. L. Joyner.** 1988. Expression patterns of the homeo box-containing genes En-1 and En-2 and the proto-oncogene int-1 diverge during mouse development. Genes Dev. **2:**1736–1744.
5. **Edwards, M. K. S., and M. W. McBurney.** 1983. The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. Dev. Biol. **98:**187–191.
6. **Fung, Y.-K. T., G. M. Shackleford, A. M. C. Brown, G. S. Sanders, and H. E. Varmus.** 1985. Nucleotide sequence and

expression in vitro of cDNA derived from mRNA of *int-1*, a provirally activated mouse mammary oncogene. Mol. Cell. Biol. **5**:3337–3344.

7. **Gorman, C., P. W. J. Rigby, and D. P. Lane.** 1985. Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell **42**:519–526.
8. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
9. **Graham, F. L., and A. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
10. **Herman, S. A., and J. M. Coffin.** 1986. Differential transcription from the long terminal repeats of integrated avian leukosis virus DNA. J. Virol. **60**:497–505.
11. **Herman, S. A., and J. M. Coffin.** 1987. Efficient packaging of readthrough RNA in ALV: implications for oncogene transduction. Science **236**:845–848.
12. **Ingham, P. W.** 1988. The molecular genetics of embryonic pattern formation in Drosophila. Nature (London) **335**:25–34.
13. **Ingham, P. W., N. E. Baker, and A. Martinez-Arias.** 1988. Regulation of segment polarity genes in the Drosophila blastoderm by fushi tarazu and even skipped. Nature (London) **331**:73–75.
14. **Jakobovits, A., G. M. Shackleford, H. E. Varmus, and G. R. Martin.** 1986. Two proto-oncogenes implicated in mammary carcinogenesis, int-1 and int-2, are independently regulated during mouse development. Proc. Natl. Acad. Sci. USA **83**: 7806–7810.
15. **Kawasaki, E. S., S. S. Clark, M. Y. Coyne, S. D. Smith, R. Champlin, O. N. Witte, and F. P. McCormick.** 1988. Diagnosis of chronic myeloid and acute lymphocytic leukemias by detection of leukemia-specific mRNA sequences amplified in vitro. Proc. Natl. Acad. Sci. USA **85**:5698–5702.
16. **Krimpenfort, P., R. De Jong, Y. Uematsu, Z. Dembic, S. Ryser, H. Von Boehmer, M. Steimetz, and A. Berns.** 1988. Transcription of T cell receptor beta chain is controlled by a downstream regulatory element. EMBO J. **7**:745–750.
17. **Linney, E., B. Davis, J. Overhauser, E. Chao, and H. Fan.** 1984. Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells. Nature (London) **308**:470–472.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific cleavages. Methods Enzymol. **65**:499–560.
20. **Melton, D. A., P. A. Kreig, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
21. **Myers, R. M., Z. Larin, and T. Maniatis.** 1985. Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science. **230**:1242–1246.
22. **Nusse, R.** 1986. The activation of cellular oncogenes by retroviral insertion. Trends Genet. **2**:244–247.
23. **Nusse, R., A. Van Ooyen, D. Cox, Y. K. T. Fung, and H. E. Varmus.** 1984. Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. Nature (London) **307**:131–136.
24. **Nusse, R., and H. E. Varmus.** 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell **31**:99–109.
25. **Rijsewijk, F., M. Schuermann, E. Wagenaar, P. Parren, D. Weigel, and R. Nusse.** 1987. The Drosophila homologue of the mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell **50**:649–657.
26. **Rijsewijk, F., L. Van Deemter, E. Wagenaar, A. Sonnenberg, and R. Nusse.** 1987. Transfection of the int-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J. **6**:127–131.
27. **Rijsewijk, F. A. M., M. Van Lohuizen, A. Van Ooyen, and R. Nusse.** 1986. Construction of a retroviral cDNA version of the int-1 mammary oncogene and its expression in vitro. Nucleic Acids Res. **14**:693–702.
28. **Robinson, H. L., and G. C. Gagnon.** 1986. Patterns of proviral insertion and deletion in avian leukosis virus-induced lymphomas. J. Virol. **57**:28–36.
29. **Rubin, M. R., L. E. Toth, M. D. Patel, P. D'Eustachio, and M. C. Nguyen-Huu.** 1986. A mouse homeo box gene is expressed in spermatocytes and embryos. Science **233**:663–667.
30. **Schuuring, E., L. van Deemter, H. Roelink, and R. Nusse.** 1989. Transient expression of the proto-oncogene *int-1* during differentiation of P19 embryonal carcinoma cells. Mol. Cell. Biol. **9**:1357–1361.
31. **Serfling, E.** 1989. Autoregulation—a common property of eukaryotic transcription factors? Trends Genet. **5**:131–133.
32. **Shackleford, G. M., and H. E. Varmus.** 1987. Expression of the proto-oncogene int-1 is restricted to postmeiotic male germ cells and the neural tube of mid-gestational embryos. Cell **50**:89–95.
33. **St. Arnaud, R., J. Craig, M. W. McBurney, and J. Papkoff.** 1989. The int-1 proto-oncogene is transcriptionally activated during neuroectodermal differentiation of P19 mouse embryonal carcinoma cells. Oncogene **4**:1077–1080.
34. **Swanstrom, R., R. C. Parker, H. E. Varmus, and J. M. Bishop.** 1983. Transduction of a cellular oncogene: the genesis of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA **80**:2519–2523.
35. **Tsukamoto, A. S., R. Grosschedl, R. C. Guzman, T. Parslow, and H. E. Varmus.** 1988. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell **55**:619–625.
36. **Van Ooyen, A., V. Kwee, and R. Nusse.** 1985. The nucleotide sequence of the human int-1 mammary oncogene; evolutionary conservation of coding and noncoding sequences. EMBO J. **4**:2905–2909.
37. **Van Ooyen, A., and R. Nusse.** 1984. Structure and nucleotide sequence of the putative mammary oncogene int-1: proviral insertions leave the protein-encoding domain intact. Cell **39**: 233–240.
38. **Varmus, H. E.** 1984. The molecular genetics of cellular oncogenes. Annu. Rev. Genet. **18**:553–612.
39. **Wasylyk, B., C. Wasylyk, P. Augereau, and P. Chambon.** 1983. The SV40 72 bp repeat preferentially potentiates transcription starting from proximal natural or substitute promoter elements. Cell **32**:503–514.
40. **Westaway, D., P. A. Goodman, C. A. Mirenda, M. P. McKinley, G. A. Carlson, and S. B. Prusiner.** 1987. Distinct prion proteins in short and long Scrapie incubation period mice. Cell **51**: 651–662.
41. **Wilkinson, D. G., J. A. Bailes, and A. P. McMahon.** 1987. Expression of the proto-oncogene int-1 is restricted to specific neural cells in the developing mouse embryo. Cell **50**:79–88.
42. **Wolgemuth, D. J., C. M. Viviano, E. Gizang-Ginsberg, M. A. Frohman, A. L. Joyner, and G. R. Martin.** 1987. Differential expression of the mouse homeobox-containing gene Hox-1.4 during male germ cell differentiation and embryonic development. Proc. Natl. Acad. Sci. USA **84**:5813–5817.