Development 1993 Supplement, 95-103 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Wingless, the *Drosophila* homolog of the proto-oncogene *Wnt*-1, can transform mouse mammary epithelial cells

Naren R. Ramakrishna and Anthony M. C. Brown*

Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York 10021, USA

*Author for correspondence

SUMMARY

The segment polarity gene *wingless* is the *Drosophila* ortholog of mouse *Wnt*-1, a proto-oncogene capable of causing transformation of mammary epithelial cells. These two genes presently represent the best studied members of the Wnt gene family. To evaluate the functional significance of the sequence conservation between *wingless* and *Wnt*-1, we have examined the effects of expressing the *Drosophila* gene in mouse mammary epithelial cell lines. *wingless* induced morphological transformation, focus formation, and mitogenesis in confluent cultures of these cells, with resulting phenotypes comparable to those obtained with mouse *Wnt*-1. In addition, RAC311c mammary cells expressing *wingless* were tumorigenic, indicating that the *Drosophila* gene is capable of inducing full neoplastic transformation. In cell co-culture experiments, *wingless* caused transformation via a paracrine mechanism, consistent with the extracellular location of its product and its proposed mechanism of action in *Drosophila* embryos. Our results indicate that *wingless* is functionally analogous to *Wnt*-1 in these mammary cell transformation assays and imply a striking conservation in the properties of the two gene products and their mechanisms of action.

Key words: *Drosophila*, proto-oncogene, segment polarity, *wingless*, *Wnt*-1

INTRODUCTION

The Wnt gene family constitutes an important group of structurally related genes implicated in regulating a wide variety of developmental processes in both vertebrates and invertebrates (for reviews see Nusse and Varmus, 1992; McMahon, 1992; Moon, 1993). The genes encode secreted proteins which are thought to act as extracellular signaling factors. At least 10 different Wnt genes are so far known in the mouse, 3 or more in *Drosophila*, and additional members of the family have been described in several other species (Nusse and Varmus, 1992; McMahon, 1992; Moon, 1993; Sidow, 1992; Eisenberg et al., 1992; Russell et al., 1992). The best studied members of the family are the mouse proto-oncogene *Wnt*-1 and its *Drosophila* homolog, the segment polarity gene *wingless* (Nusse and Varmus, 1982; Rijsewijk et al., 1987a). Much of our present knowledge of *Wnt* gene function has accrued from analysis of these two genes in disparate biological systems.

Wnt-1 was first identified as a murine oncogene activated in carcinomas induced by the mouse mammary tumor virus (MMTV), although the gene normally functions in the embryonic central nervous system (Nusse and Varmus, 1992; McMahon, 1992). In mid-gestational mouse embryos *Wnt*-1 is expressed in a spatially restricted pattern within the neural tube (Shackleford and Varmus, 1987; Wilkinson et al., 1987), and the phenotypes of animals homozygous for null alleles demonstrate that *Wnt*-1 plays an essential role in development of the fetal midbrain and cerebellum (Thomas and Capecchi, 1990; McMahon and Bradley, 1990; Thomas et al., 1991; McMahon et al., 1992). *Wnt*-1 encodes cysteine-rich secreted glycoproteins of $41\text{-}44\times10^3$ M_r, which in cell culture are found predominantly associated with the extracellular matrix (ECM) or cell surface (Brown et al., 1987; Papkoff et al., 1987; Papkoff, 1989; Bradley and Brown, 1990; Papkoff and Schryver, 1990) and which are believed to function in the manner of growth or differentiation factors (see Nusse and Varmus, 1992, for review).

Mouse mammary tumors induced by MMTV frequently contain proviral DNA insertions at the *Wnt*-1 locus (Nusse and Varmus, 1982) and these insertions activate expression of wild-type Wnt-1 protein within tissue in which the gene is normally silent (Nusse et al., 1984; van Ooyen et al., 1985). The consequences of this have been duplicated experimentally in transgenic mice designed to express *Wnt*-1 in their mammary glands: such mice rapidly develop mammary hyperplasia and subsequently carcinomas (Tsukamoto et al., 1988). In addition, the transforming potential of *Wnt*-1 has been demonstrated in murine cell culture systems. Expression of *Wnt*-1 in the mammary epithelial cell line C57MG causes morphological transformation and an apparent loss of contact-inhibition of cell growth (Brown et al., 1986). As a result, cultures grow to higher final cell densities and fail to become quiescent at confluence. Similar

effects of *Wnt*-1 have been described in the mammary cell line RAC311c, which can be made tumorigenic by expression of the gene (Rijsewijk et al., 1987b). In contrast, no manifest phenotypic effects of *Wnt*-1 have been observed in other cell lines so far tested, including several fibroblast lines, HeLa and MDCK cells (Brown et al., 1986; Jue et al., 1992; Mason et al., 1992). This cell type specificity of *Wnt*-1 transformation has recently been exploited to demonstrate that fibroblasts expressing *Wnt*-1, although not themselves phenotypically altered, are able to induce transformation of surrounding C57MG cells in co-cultures (Jue et al., 1992). This effect presumably results from secreted Wnt-1 protein acting in a paracrine manner.

Wnt-1 displays a striking degree of phylogenetic conservation and orthologs have so far been described in human, mouse, reptiles, amphibians, bony and cartilaginous fishes, echinoderms, and *Drosophila* (Nusse et al., 1984; van Ooyen et al., 1985; Noordermeer et al., 1989; Molven et al., 1991; Busse et al., 1990; Rijsewijk et al., 1987a; Sidow, 1992). The *Drosophila* ortholog of *Wnt*-1 is the segment polarity gene *wingless*, a gene required for correct pattern formation within the embryonic body segments and imaginal discs (Couso et al., 1993; Struhl and Basler, 1993; Babu, 1977; Baker, 1988a; Baker, 1987; Cabrera et al., 1987; Rijsewijk et al., 1987a; Nusslein-Volhard and Wieschaus, 1980). In the embryonic ectoderm, *wingless* is expressed in narrow stripes of epidermal cells at the posterior boundary of each parasegment (Baker, 1988b; Baker, 1987), and there is both genetic and immunohistochemical evidence that it functions in intercellular signaling to modulate the phenotype and developmental fate of neighboring cells (Wieschaus and Riggleman, 1987; Morata and Lawrence, 1977; Martinez-Arias et al., 1988; DiNardo et al., 1988; Riggleman et al., 1990; Dougan and DiNardo, 1992; van den Heuvel et al., 1989; Gonzalez et al., 1991). Like mouse Wnt-1, Wingless protein is secreted and may be associated with extracellular matrix or cell surfaces in the embryo (van den Heuvel et al., 1989; Gonzalez et al., 1991).

At the amino acid level, Wingless protein and mouse Wnt-1 are 54% identical (Fung et al., 1985; Rijsewijk et al., 1987a). Both are cysteine-rich proteins and all 23 cysteine residues in both proteins are found in equivalent positions (Rijsewijk et al., 1987a). Sequence conservation extends over most of the length of the predicted gene products, with two exceptions. The amino-terminal domains show no significant sequence identity, although both contain secretory signal peptides, and the predicted Wingless protein contains an internal 93 amino acid insertion not present in mouse Wnt-1 (Fung et al., 1985; Rijsewijk et al., 1987a).

Like *Wnt*-1, many other vertebrate proto-oncogenes display a high degree of evolutionary conservation and homologs of several such genes have been identified in *Drosophila* (reviewed by Shilo, 1987; Hoffmann, 1989). The ability to perform genetic analysis of these genes in *Drosophila* offers substantial promise of better understanding of the mechanisms by which their mammalian counterparts operate. In the case of *Wnt*-1/*wingless*, candidate genes in the *wingless* signaling or response pathway have already emerged from extensive analysis of segment polarity (Ingham, 1991; Peifer and Bejsovec, 1992). However, the relevance of these studies to mammalian *Wnt*-1 depends in part on the extent to which the sequence homology reflects conservation in the properties of the *Wnt*-1 and *wingless* gene products. In this paper we evaluate the action of *wingless* in cell culture assays commonly used to study *Wnt*-1 function. We show that the *Drosophila* gene, like mouse *Wnt*-1, is capable of causing transformation and mitogenesis of mouse mammary cells and of converting them to a tumorigenic phenotype. Despite the large phylogenetic distance between Diptera and mammals, our results indicate that the *wingless* and *Wnt*-1 gene products share a common mechanism of action and are functionally equivalent in these transformation and oncogenesis assays.

MATERIALS AND METHODS

Cell culture and retrovirus vectors

Clonal derivatives of the mouse mammary epithelial cell lines C57MG (Vaidya et al., 1978) and RAC311c (Rijsewijk et al., 1987b) were chosen for their homogeneous flat morphology at confluence and were maintained as previously described for C57MG (Jue et al., 1992).

To construct pMV*wg*, a 1.9 kb fragment of *wingless* cDNA (nucleotides 284-2181; Rijsewijk et al., 1987a) was cloned into the MSV-based retroviral vector plasmid pMV7 (Kirschmeier et al., 1988). pMV*Wnt*-1, an equivalent construct containing mouse *Wnt*-1 cDNA, has been described previously (Jue et al., 1992). Helper-free virus stocks were produced by a two-step procedure in which pMV*wg*, pMV*Wnt*-1, and pMV7 were first introduced into the ecotropic retroviral packaging cell line E86 (Markowitz et al., 1988b) and stable transfectants were selected in G418 (Brown and Scott, 1987). Virus stocks harvested from pooled populations of resistant colonies were then used to infect the amphotropic packaging cell line AM12 (Markowitz et al., 1988a), again with G418 selection. Virus was harvested both from clonal cultures of infected AM12 cells and from pooled populations. Titers were determined from *neo* transduction efficiencies upon infection of Rat-2 cells and selection of colonies in G418 (Brown and Scott, 1987). Absence of replication-competent helper virus was verified by assaying the *neo*-transducing potential of supernatants from infected Rat-2 and C57MG cells.

Focus assays were performed as described previously (Brown et al., 1986). Foci were first visible 1-2 days after the cells reached confluence and were counted and photographed 1-2 days later. Focus-forming units (FFU) were determined from duplicate infections at three different virus dilutions. Cell co-culture assays were performed as described by Jue et al. (1992).

Measurements of cell density and DNA synthesis

C57MG cells infected with MV7, MV*wg* or MV*Wnt*-1 were plated at a density of 7.5×10^4 cells per 6 cm dish and grown to confluence over a 5 day period. Using a hematocytometer, total cell numbers in triplicate dishes were determined on three successive days, beginning on the first day that the cultures appeared confluent. Measurements of DNA synthesis were performed on cells plated in parallel with the above. On the day the cultures first reached confluence, methyl-[^{3}H]thymidine (Amersham) was added to the medium at a final concentration of 10 μCi/ml. 24 hours later the cells were washed in PBS and fixed in 3.7% formaldehyde. The monolayers were then washed in 5% TCA, rinsed, and coated with Kodak NTB-2 photographic emulsion. After 2 days exposure, numbers of labelled nuclei per unit area were counted using an inverted microscope.

Immunofluorescence and immunoblotting

For indirect immunofluorescence, a polyclonal rat serum raised

against a Wingless fusion polypeptide (Gonzalez et al., 1991) was kindly provided by A. Martinez Arias (University of Cambridge, UK). The serum was preadsorbed against methanol-fixed C57MG/MV7 cells. Cells were grown on glass coverslips, fixed for 10 minutes in methanol at –30°C, and incubated with a 1:100 dilution of preadsorbed antiserum for 1 hour at 37°C. Secondary antibody was goat anti-rat IgG conjugated to Texas Red (Jackson Immunoresearch). For staining of ECM-associated Wingless protein, cells were detached by incubating for 5 minutes at 37°C in 1 mM EDTA in PBS and the coverslips were then treated with fixative and immunostained as above. For staining of cell surface-associated Wingless protein, cells were grown on poly-D-lysine-coated coverslips and incubated with *wingless* antiserum at 1:100 dilution for 12 hours at 4°C prior to fixation and secondary antibody staining as described above.

For immunoblot analysis, conditioned culture medium was pre-cleared at 1000 *g* and proteins sedimented at 100,000 *g* for 3 hours in the presence of protease inhibitors as described by Jue et al. (1992). Where mentioned, 200 μg/ml heparin (from porcine intestinal mucosa, Sigma) was added to the culture medium for 48 hours. After electrophoresis and transfer to nitrocellulose (Bradley and Brown, 1990), Wingless products were detected using a 1:1500 dilution of rabbit anti-Wingless serum (a gift from R. Nusse, Stanford University) followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega) and were visualized as previously described (Bradley and Brown, 1990).

RESULTS

A retrovirus expressing *wingless* induces transformed foci in C57MG mammary cells

To express *wingless* and *Wnt*-1 efficiently in mammalian cells, we used the recombinant retrovirus vectors MV*wg* and MV*Wnt*-1 derived from the murine sarcoma virus based vector pMV7 (Kirschmeier et al., 1988). These vectors express *wingless* or *Wnt*-1 cDNA from the viral long terminal repeat promoter, and the selectable marker *neo* from an internal thymidine kinase gene promoter. Helper-free stocks of these viruses, and of the parental vector MV7, were obtained from retroviral packaging cell lines and their *neo* transducing efficiency was determined as a measure of overall virus titer.

We first tested the transforming potential of *wingless* in mouse mammary epithelial cells by means of a focus assay in which C57MG cells were infected with MV*wg* or MV*Wnt*-1 at low multiplicity of infection and allowed to grow to confluence without selection. After they reached confluence, cultures infected with either virus showed distinct foci of morphologically transformed refractile cells within the otherwise flat cuboidal cell monolayer (Fig. 1A-C). The focus-forming efficiency of MV*wg* virus was approximately five-fold lower than that of MV*Wnt*-1 (Table 1), and the foci induced were generally smaller and took 1-2 days longer to develop than those induced by MV*Wnt*-1. Nevertheless, the numbers of foci were proportional to the quantities of MV*wg* virus applied, and the lack of significant focus formation induced by the control virus MV7 confirmed that the transformed foci induced by MV*wg* were *wingless*-dependent. Staining of nuclear DNA in these cultures showed that the foci constitute regions of increased cell density as well as transformed morphology (data not shown), suggesting that *wingless* has a mitogenic effect on these cells at confluence as well as a capacity to induce morphological transformation.

Table 1. Focus-forming abilities of retrovirus stocks

Virus stock	FFU/ml	CFU/ml	FFU/CFU
MV7	2.3	3.3×10^5	0%
MV*wg*	5.8×10^3	9.2×10^4	6%
MV*Wnt*-1	3.1×10^4	9.9×10^4	32%

Relative focus forming ability of each virus stock is expressed as the ratio of focus forming units (FFU) to G418-resistant colony forming units (CFU) per ml.

C57MG cells expressing *wingless* are morphologically transformed and grow to higher densities

We also examined the phenotype of C57MG cells that were selected for infection with the above virus stocks and grown as pooled populations of G418-resistant colonies. 2 days after reaching confluence, cultures infected with the control virus MV7 formed a monolayer of flat cuboidal cells similar to uninfected cultures (Fig. 1D). Those infected with MV*wg*, however, were morphologically transformed and indistinguishable from MV*Wnt*-1-infected cultures with respect to their refractile and disordered appearance (Fig. 1E,F). To assess the mitogenic potential of *wingless* expression in confluent C57MG cells, we first measured the maximal cell densities achieved in the infected cultures: those infected with MV*wg* grew to 3- to 4-fold higher cell densities than MV7-infected controls and their final densities were equivalent to those of *Wnt*-1-transformed cells (Fig. 2A). We next examined DNA synthesis in confluent cultures by measuring [^{3}H]thymidine incorporation into nuclei. Despite their higher cell densities, MV*wg*-infected cultures were substantially more active in DNA synthesis than control cells, again implying that expression of *wingless* is mitogenic in confluent cultures of C57MG cells (Fig. 2B).

RAC311c mammary cells expressing *wingless* are tumorigenic

Taken together, the above results indicate that *wingless* can induce transformation of C57MG cells with a resulting phenotype equivalent to that obtained by expression of *Wnt*-1 (Brown et al., 1986). Similar morphological changes were also observed when *wingless* was expressed in RAC311c cells, another mammary line that can be transformed by *Wnt*-1 (Fig. 1G-I; Rijsewijk et al., 1987b). In contrast, no phenotypic effects of *wingless* were seen in Rat-2 cells (data not shown), consistent with the known cell-type specificity of *Wnt*-1 transformation (Jue et al., 1992).

Unlike C57MG cells, RAC311c cells can be converted to a tumorigenic phenotype by expression of mouse *Wnt*-1 (Brown et al., 1986; Rijsewijk et al., 1987b). To determine whether *Drosophila wingless* could substitute for the mouse gene in this oncogenicity assay, pooled populations of infected RAC311c cells were injected subcutaneously into athymic *nu/nu* mice. While no tumors were detected at sites injected with MV7-infected control cells, more than 50% of the sites receiving MV*wg*-infected cells produced tumors within 12 weeks (Table 2). The frequency and latency of these *wingless*-induced tumors were not significantly

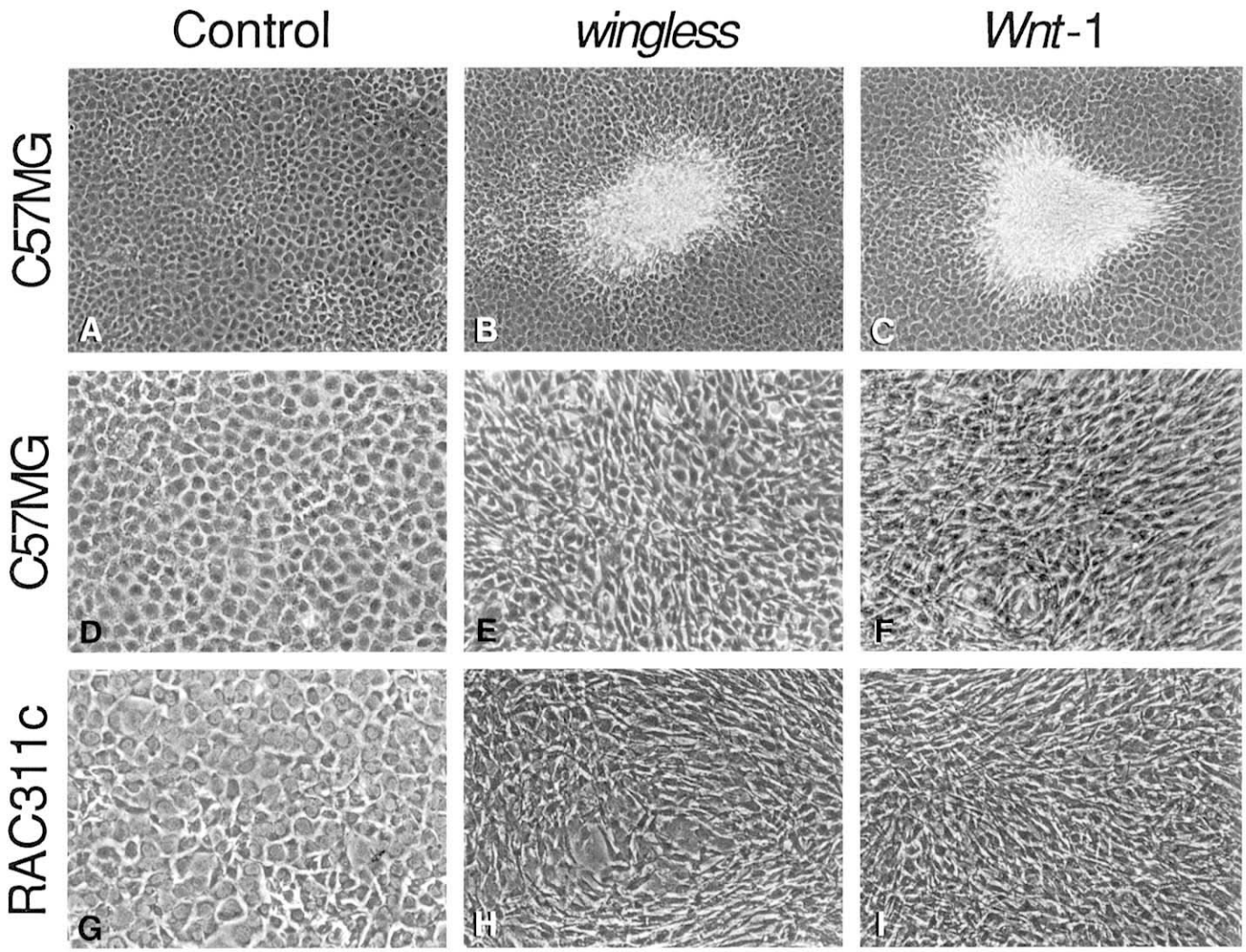

Fig. 1. Drosophila *wingless* can transform mouse mammary epithelial cells. (A-C) Focus assay on C57MG cells. Cells were infected with low titres of recombinant retrovirus vectors MV*wg* and MV*Wnt*-1 and left to continue growing in the same dishes. (A) Confluent monolayer of uninfected C57MG cells. (B) Example of a focus of refractile transformed cells induced by MV*wg*. (C) Focus induced by MV*Wnt*-1. (D-I) Morphology of C57MG cells (D-F) or RAC311c cells (G-I) selected for infection with the control virus MV7 (D and G), MV*wg* (E and H), or MV*Wnt*-1 (F and I). Cultures are shown 2 days after reaching confluence. The morphology of populations infected with the MV7 virus, which contains the *neo* gene only, is similar to that of uninfected cells, while cells infected with MV*wg* or MV*Wnt*-1 are refractile, elongated, and disordered. D-I were photographed at higher magnification than A-C.

different from those observed with RAC311c cells infected with MV*Wnt*-1 (Table 2).

Detection of Wingless protein in MV*wg*-infected cell cultures

To confirm that the MV*wg* virus used in these experiments directed synthesis of Wingless protein, and to examine the distribution of the protein, we analyzed *MVwg*-infected cells by indirect immunofluorescence using an anti-Wingless antiserum (Gonzalez et al., 1991). In methanol-fixed C57MG/MV*wg* cells we observed a staining pattern consistent with localization of Wingless antigen to the endoplasmic reticulum and Golgi (Fig. 3A). As well as this intracellular staining, in some fields we noticed diffuse extracellular staining beyond the cell boundaries. To examine this further, intact cells were detached from coverslips by treatment with EDTA and the extracellular matrix material remaining on the coverslips was processed for immunostaining. This revealed extensive cloudy staining in the matrix which was specific for cells expressing *wingless*. If cells were detached when at low density, the patterns of staining often resembled

Table 2. Tumorigenicity of infected RAC 311c cells

Cell line	Time of tumor onset (weeks) 6—8	8—10	10—12	Tumors/ total sites (%)
Rac 311c/MV7	0	0	0	0/15 (0)
RAC311c/MV*wg*	2	6	4	12/22 (55)
RAC311c/MV*Wnt*-1	2	4	1	7/11 (64)

5×10^6 cells were injected subcutaneously into syngeneic BALB/c *nu/nu* mice and tumors of ≥0.3 cm diameter appearing over subsequent 2 week periods were recorded.

blurred 'footprints' of cells, as if corresponding to regions where cells had previously adhered to the substrate (Fig. 3B). This material did not stain with antibodies to vinculin or α-actinin (data not shown) and cellular debris was not visible in these regions under phase contrast illumination.

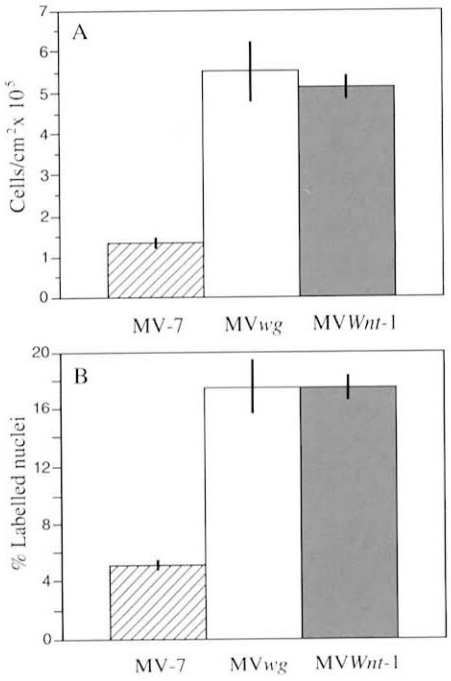

Fig. 2. Expression of *wingless* in C57MG cells is mitogenic in confluent cultures. (A) Cells infected with MV*wg* grow to higher saturation densities than control cells. C57MG cells infected with MV7, MV*wg*, or MV*Wnt*-1 were grown to confluence and their final cell densities determined. The apparent mitogenic effect of *wingless* in these cells is quantitatively similar to that of mouse *Wnt*-1. (B) Incorporation of [^{3}H]thymidine at confluence shows that C57MG/MV*wg* cells continue to synthesize DNA under conditions which induce quiescence of control cultures. Confluent cultures were labeled with [^{3}H]thymidine for 24 hours and the numbers of labeled and unlabeled nuclei were counted after autoradiography.

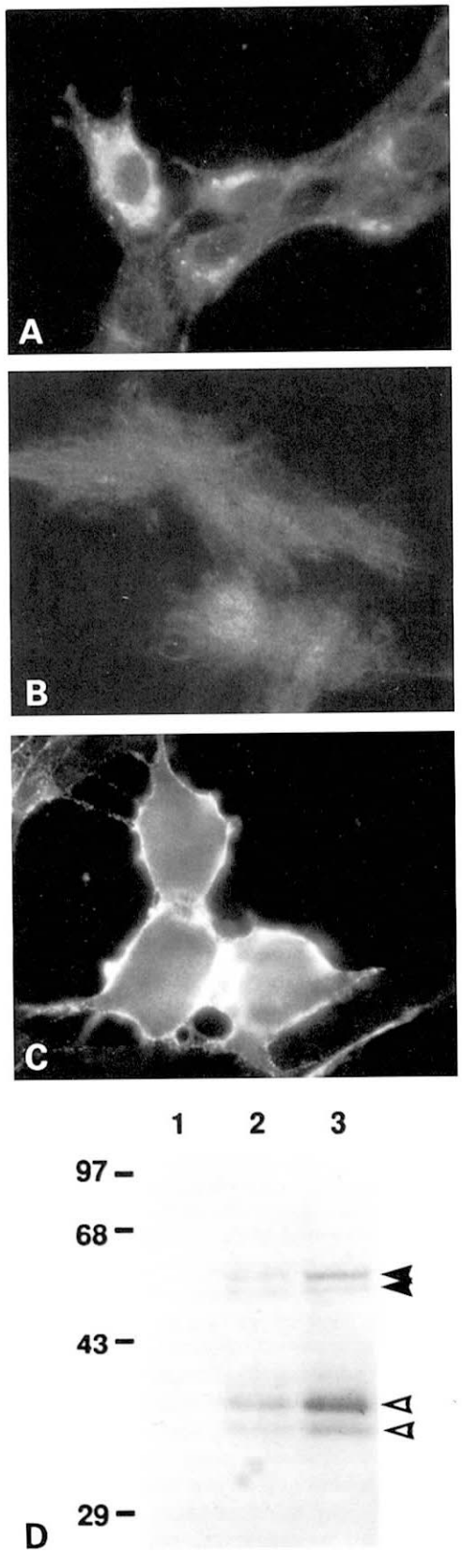

Fig. 3. Wingless protein is secreted from C57MG cells infected with MV*wg*. (A-C) Indirect immunofluorescence shows Wingless in the secretory pathway and associated with the extracellular matrix and cell surface. (A) C57MG/MV*wg* cells were fixed in cold methanol and stained with rat anti-Wingless antiserum. The staining pattern is consistent with localization to the endoplasmic reticulum and Golgi. (B) Staining of cover slips after removal of C57MG/MV*wg* cells shows secreted Wingless antigen associated with extracellular matrix. (C) Staining of cells on lysine-coated coverslips without fixation or permeabilization shows cell-surface associated wingless antigen. No significant staining was detected in cultures of control C57MG/MV7 cells treated as in A-C (data not shown). (D) Wingless proteins sedimented from cell culture medium. Conditioned medium was harvested from cultures of control C57MG/MV7 cells grown with added heparin (lane 1), and C57MG/MV*wg* cells grown without (lane 2) or with added heparin (lane 3). Proteins sedimented from 2 ml of medium by centrifugation at 100,000 ***g*** were analyzed on western blots using rabbit anti-Wingless antiserum. Filled arrowheads mark proteins of approximately 51 and 53×10^3 M_r which are close to the expected size for full length Wingless products. Smaller Wingless polypeptides of 35 and 37×10^3 M_r are marked by open arrowheads.

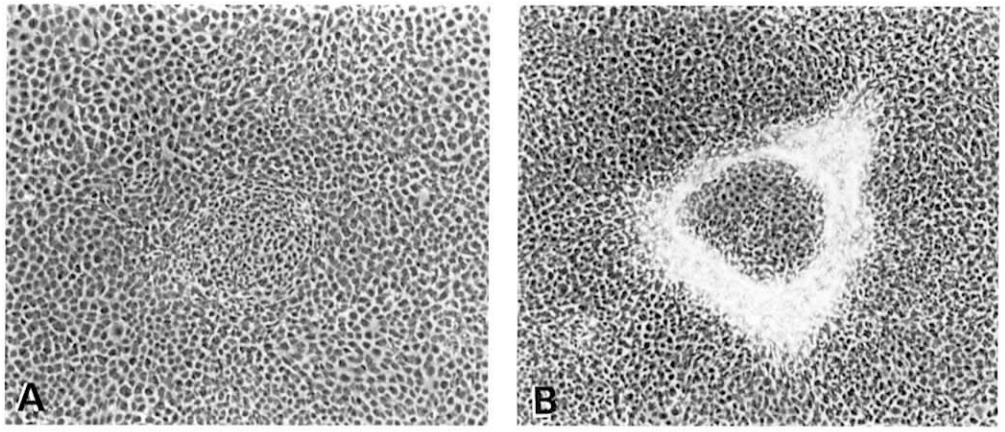

Fig. 4. Rat-2 cells infected with MV*wg* induce transformation of neighboring C57MG cells in co-culture experiments. A small number of infected Rat-2 fibroblasts were plated together with an excess of uninfected C57MG mammary epithelial cells and the mixture grown to confluence. (A) Co-culture showing a colony of control Rat-2/MV7 cells at center surrounded by the C57MG monolayer. (B) Similar co-culture with colony of Rat-2/MV*wg* at center. A refractile ring of morphologically transformed C57MG cells surrounds the fibroblasts expressing *wingless*.

These results therefore indicate that a proportion of the Wingless product secreted from the cells is bound to ECM material in the immediate vicinity. This finding has been confirmed by immunoblot analysis of ECM fractions (data not shown). In addition, we performed staining of unfixed cell cultures and observed Wingless antigen associated with the cell surface or pericellular matrix (Fig. 3C). Similar patterns of intracellular and extracellular staining were also seen using RAC311c/MV*wg* cells or Rat-2/MV*wg* (data not shown).

To seek evidence of any Wingless protein released into the cell culture medium, we subjected conditioned medium from C57MG/MV*wg* cells to centrifugation at 100,000 ***g*** and were able to sediment Wingless products under these conditions (Fig. 3D). Their abundance in this pelleted fraction was increased slightly by including heparin in the culture medium (lanes 2 and 3). Immunoblot analysis of these proteins showed that a significant proportion had undergone proteolytic cleavage, although putative full length species of $51\text{-}53\times10^3$ M_r were also detected (Fig. 3D).

Wingless can induce transformation via a paracrine mechanism

In *Drosophila* there is extensive evidence suggesting that *wingless* acts in intercellular signaling, and we wished to investigate this in the present cell culture system. Since *wingless* does not induce transformation of Rat-2 cells, we tested the ability of the gene to function in the paracrine transformation assay described by Jue et al. (1992). Rat-2 cells infected with either MV*wg* or MV7 were co-cultured with a large excess of uninfected C57MG cells and the mixture allowed to grow to confluence. In control cultures, the Rat-2/MV7 derivatives formed discrete colonies scattered throughout the C57MG cell monolayer (Fig. 4A). In co-cultures containing Wingless-expressing cells, however, we observed rings of morphologically transformed C57MG cells surrounding the majority of Rat-2/MV*wg* colonies (Fig. 4B). Thus, as a result of expressing Wingless, the Rat-2/MV*wg* fibroblasts were able to induce transformation of neighboring mammary cells, some of which were located several cell diameters away from Wingless-expressing cells. These results confirm that the *wingless* gene can act at a distance to affect the phenotype of neighboring cells.

DISCUSSION

Drosophila homologues of vertebrate proto-oncogenes include genes encoding transcription factors, members of the ras family of GTPases, cytoplasmic and membrane-bound kinases, transmembrane receptors, and growth factor-like molecules (Shilo, 1987; Hoffmann, 1989; Katzen et al., 1991; Pulido et al., 1992; van Lohuizen et al., 1991). Although in some cases there is evidence for conservation of transforming potential in chimeric mutant proteins (Holland et al., 1990), normal *Drosophila* gene products have not so far been shown capable of effecting transformation of mammalian cells. In this report we have used mammary cell transformation assays to evaluate the functional significance of sequence conservation between the *Drosophila* segment polarity gene *wingless* and its murine homolog, the proto-oncogene *Wnt*-1 (Rijsewijk et al., 1987a). We have shown that *wingless* can induce morphological transformation and mitogenic effects in mouse mammary cell lines with a resulting phenotype comparable to that seen with *Wnt*-1, that the transforming potential of the two genes show similar cell-type specificities, and that RAC311c mammary epithelial cells expressing *wingless* are tumorigenic in mice. By these criteria, therefore, *wingless* can act as an oncogene in certain mammalian cell lines.

As well as qualitative similarities in phenotype, the effects of expressing *wingless* and *Wnt*-1 in mammary epithelial cells were quantitatively similar in assays of mitogenesis in confluent cell cultures, maximum cell densities, and tumorigenic potential. *wingless* was approximately 5-fold less efficient than *Wnt*-1 in focus assays on unselected C57MG cell monolayers, however. The latter assays, which probably depend on a combination of autocrine and paracrine transformation, are likely to be the most sensitive in revealing differences in transforming potential. We also noted that *wingless* was somewhat less efficient than *Wnt*-1 at inducing paracrine transformation in co-culture assays. It remains to be determined whether these differences in

activity are intrinsic to the two proteins, or reflect differential efficiencies of their translation or secretion in these cells.

Of the 370 amino acids in mouse Wnt-1 protein, 54% are conserved in the Wingless product. The human *WNT*-2 gene has also been shown to cause transformation of C57MG cells (Blasband et al., 1992), and when this sequence is taken into account, 122 residues (33% of those in Wnt-1) are common to all three gene products. The conserved amino acids extend over most of the length of the proteins, suggesting that sequences responsible for transformation may not be confined to a single discrete domain. The most striking features of the conserved sequence are the 22 cysteine residues whose relative positions are nearly identical in all three proteins. Assuming that most of these are involved in disulfide bonding, it is likely that a specific tertiary structure is critically important for Wnt protein function. The numerous cysteines are also conserved in most members of the *Wnt* gene family that have been sequenced to date (Nusse and Varmus, 1992; McMahon, 1992; and refs. therein). The structure they dictate is clearly not sufficient for transformation, however, since mammary cell transformation is not a universal property of all *Wnt* genes (J. Kitajewski, personal communication).

The protein products of *wingless* and *Wnt*-1 show similar secretory properties in the cells used in these experiments. We have previously shown that secreted Wnt-1 protein is associated with the extracellular matrix of cultured cell lines (Bradley and Brown, 1990), and Papkoff and Schryver (1990) have reported Wnt-1 associated with the cell surface or pericellular matrix. Using antibodies to visualize *wingless* products by immunofluorescence, we have shown here that Wingless protein is present both at the cell surface and in the ECM of C57MG cell cultures. In addition, we detected Wingless product in the conditioned culture medium by centrifuging the medium and analyzing the pellet by immunoblotting. The ease with which this fraction could be sedimented, however, suggests that it is not freely soluble and instead may be present within molecular aggregates, possibly including ECM components not bound to the substratum. One way to explain the distribution of Wingless in these cultures would be to propose that the secreted protein itself is bound to another molecule, such as a proteoglycan, which may exist both as a surface-bound moiety and in a form released into the ECM and culture medium.

The extracellular location of Wingless protein in these mammalian cell cultures is broadly consistent with the distribution of Wingless antigen observed in *Drosophila* embryos, which can be detected at 1-3 cell diameters distant from cells expressing *wingless* RNA (van den Heuvel et al., 1989; Gonzalez et al., 1991). More specifically, our data add support to the conclusions of van den Heuvel et al. (1989) that a proportion of Wingless antigen is associated with the ECM in *Drosophila*. In contrast, a recent study of the distribution of Wingless protein expressed in *Xenopus* oocytes and embryos found most of the protein to be intracellular and a secreted form was not demonstrated except in the presence of high concentrations of the anionic compound suramin (Chakrabarti et al., 1992). The reasons for this apparent discrepancy are unclear but may be related to the very high level of transient expression achieved in the oocyte system, together with the propensity of Wnt-1 protein to be retained in the endoplasmic reticulum when overexpressed (Papkoff, 1989; Kitajewski et al., 1992).

In view of the secretory nature of Wingless in transformed C57MG and RAC311c cell cultures, the protein presumably acts as an autocrine factor in this system. Although in *Drosophila* the gene may act in an autocrine manner in certain cells, there is also extensive evidence that the Wingless product acts in the embryo as a paracrine factor (reviewed by Ingham, 1991; Peifer and Bejsovec, 1992). For example, *wingless* expression in the epidermis of early embryos is necessary for maintenance of *engrailed* expression in neighboring cells, and Wingless protein can be detected within cells adjacent to those in which it is synthesized (Martinez Arias et al., 1988; DiNardo et al., 1988; van den Heuvel et al., 1989; Gonzalez et al., 1991; Heemskerk et al., 1991). In what is apparently a combination of autocrine and paracrine effects, *wingless* causes modulation of Armadillo protein in broad stripes of cells including and surrounding those expressing *wingless* RNA (Riggleman et al., 1990). In addition, clones of *wingless* mutant cells behave in a non-autonomous manner in genetic mosaics (Wieschaus and Riggleman, 1987; Morata and Lawrence, 1977). All these data suggest that *wingless* normally acts in intercellular signaling and the mammary cell transformation assays described here provided an opportunity to test this notion experimentally. Although colonies of Rat-2 fibroblasts expressing Wingless protein are not themselves ostensibly altered, when co-cultured with C57MG mammary epithelial cells they were able to induce transformation of the surrounding mammary cells (Fig. 5). Like mouse *Wnt*-1, therefore, the *wingless* gene is able to elicit transformation via a paracrine mechanism (Jue et al., 1992). A paracrine effect of wingless has also recently been demonstrated in co-cultures of *Drosophila* embryonic cells (Cumberledge and Krasnow, 1993). Collectively these cell culture assays strongly support models of Wingless as an intercellular signaling factor.

In causing transformation of mammary cells, the products of both *wingless* and *Wnt*-1 presumably interact with the same cell surface receptors in the target cells. A similar notion is also implied by the recent finding that both genes can induce dorsal mesoderm in *Xenopus* embryos (Chakrabarti et al., 1992). Specific receptors for Wnt-1 or Wingless have yet to be identified in either mouse or *Drosophila*, but it seems likely that these too will share sequence homology, at least in their ligand-binding domains. If elements of the signaling pathways activated by these receptors are also conserved in evolution, it is possible that the growth-promoting effects induced by *wingless* in mouse mammary cells may reflect some of the normal consequences of *wingless* expression during *Drosophila* embryogenesis. In this regard it is particularly interesting that *wingless* appears to have a mitogenic effect on the developing Malpighian tubules of *Drosophila*, where the gene is normally expressed in the tubule primordia (Skaer and Martinez Arias, 1992). In the absence of *wingless* function, cells of the tubule anlage fail to proliferate, while over-expression of the gene in wild-type embryos can promote additional cell divisions in the tubules resulting in supernumerary cells (Skaer and Martinez Arias, 1992). In the epidermis, *wingless* expression is not known to be

mitogenic during the establishment of segment polarity, although in the absence of *wingless* function there is significant cell death accompanying the deletion of specific pattern elements within the body segments (Perrimon and Mahowald, 1987; Klingensmith et al., 1989). Further analysis will be required to determine whether the mitogenic and transforming functions of *wingless* in mammalian cells depend on the same protein domain(s) required for normal *wingless* function in *Drosophila*.

In summary, our results demonstrate that the ability of *Wnt*-1 to cause transformation of mammary epithelial cell lines has been conserved in evolution of the gene from *Drosophila* to mouse. Despite their sequence divergence, the products of *wingless* and mouse *Wnt*-1 show similar biochemical properties and can act via a common mechanism that presumably involves specific cell surface receptors. The conservation of transforming potential demonstrated here emphasizes the validity of studying oncogene homologs in *Drosophila*, in which genetic analysis may identify additional components of signaling pathways relevant to vertebrate tumorigenesis. Conversely, our data imply that studies of *Wnt*-1 and its effects in cultured cells may be directly relevant to understanding the mechanism of action of Wingless. Finally, the transformation of mouse mammary cell lines provides a convenient cell culture assay for *wingless* and should facilitate biochemical and functional studies of both wild-type and mutant Wingless proteins to elucidate their roles in intercellular signaling.

We thank Alfonso Martinez Arias, Roel Nusse, and Enrique Rodriguez-Boulan for generous gifts of reagents, Roger Bradley for helpful advice, and Shall Jue for technical assistance. This work was supported by NIH grants CA47207 and CA16599, and by funds from the Pew Scholars Program. N. R. R. is supported by the Medical Scientist Training Program of Cornell University Medical College.

REFERENCES

Babu, P. (1977). Early developmental subdivisions of the wing disk in *Drosophila*. *Mol. Gen. Genet.* **151**, 289-294.

Baker, N. (1987). Molecular cloning of sequences from *wingless*, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. *EMBO J.* **6**, 1765-1773.

Baker, N. E. (1988a). Embryonic and imaginal requirements for *wingless*, a segment polarity gene in *Drosophila*. *Dev. Biol.* **125**, 96-108.

Baker, N. E. (1988b). Localization of transcripts from the *wingless* gene in whole *Drosophila* embryos. *Development* **103**, 289-298.

Blasband, A., Schryver, B. and Papkoff, J. (1992). The biochemical properties and transforming potential of human *Wnt*-2 are similar to *Wnt*-1. *Oncogene* **7**, 155-161.

Bradley, R. S. and Brown, A. M. C. (1990). The proto-oncogene *int*-1 encodes a secreted protein associated with the extracellular matrix. *EMBO J.* **9**, 1569-1575.

Brown, A. M. C., Wildin, R. A., Prendergast, T. J. and Varmus, H. E. (1986). A retrovirus vector expressing the putative mammary oncogene *int*-1 causes partial transformation of a mammary epithelial cell line. *Cell* **46**, 1001-1009.

Brown, A. M. C., Papkoff, J., Fung, Y. K. T., Shackleford, G. M. and Varmus, H. E. (1987). Identification of protein products encoded by the proto-oncogene *int*-1. *Mol. Cell. Biol.* **7**, 3971-3977.

Brown, A. M. C. and Scott, M. R. D. (1987). Retroviral vectors. In *DNA Cloning - A Practical Approach*, vol. III (ed. Glover, D.M.), pp. 189-212. Oxford/Washington D.C.: IRL Press.

Busse, U., Guay, J. and Seguin, C. (1990). Nucleotide sequence of a cDNA encoding *Wnt*-1 of the Mexican axolotl Ambystoma mexicanum. *Nucl Acids Res.* **18**, 7439.

Cabrera, C. V., Alonso, M. C., Johnston, P., Phillips, R. G. and Lawrence, P. A. (1987). Phenocopies induced with antisense RNA identify the *wingless* gene. *Cell* **50**, 659-663.

Chakrabarti, A., Matthews, G., Colman, A. and Dale, L. (1992). Secretory and inductive properties of *Drosophila wingless* protein in *Xenopus* oocytes and embryos. *Development* **115**, 355-369.

Couso, J. P., Bate, M. and Martinez Arias, A. (1993). A *wingless*-dependent polar coordinate system in *Drosophila* imaginal discs. *Science* **259**, 484-489.

Cumberledge, S., Krasnow, M. A. (1993). Intercellular signaling in *Drosophila* segment formation reconstructed in vitro. *Nature* **363**, 549-552

DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J. A. and O'Farrel, P. H. (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. *Nature* **332**, 604-609.

Dougan, S. and DiNardo, S. (1992). *Drosophila wingless* generates cell type diversity among *engrailed* expressing cells. *Nature* **360**, 347-350.

Eisenberg, L. M., Ingham, P. W., and Brown, A. M. C. (1992). Cloning and characterization of a novel *Drosophila Wnt* gene, *DWnt-5*, a putative downstream target of the homeobox gene *Distal-less*. *Dev. Biol.* **154**, 73-83.

Fung, Y. K. T., Shackleford, G. M., Brown, A. M. C., Sanders, G. S. and Varmus, H. E. (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.

Gonzalez, F., Swales, L., Bejsovec, A., Skaer, H. and Martinez Arias, A. (1991). Secretion and movement of *wingless* protein in the epidermis of the *Drosophila* embryo. *Mech. Dev.* **35**, 43-54.

Heemskerk, J., DiNardo, S., Kostriken, R. and O'Farrell, P.H. (1991). Multiple modes of *engrailed* regulation in the progression towards cell fate determination. *Nature* **352**, 404-410.

Hoffmann, F. M. (1989). Roles of Drosophila proto-oncogene and growth factor homologs during development of the fly. *Current Topics Microbiol. Immunol.* **147**, 1-29.

Holland, G. D., Henkemeyer, M. J., Kaehler, D. A., Hoffman, F. M. and Risser, R. (1990). Conservation of function of *Drosophila melanogaster abl* and murine v-*abl* proteins in transformation of mammalian cells. *J. Virol.* **64**, 2226-2235.

Ingham, P. (1991). Segment polarity genes and cell patterning within the *Drosophila* body segment. *Curr. Opin. Genet. Dev.* **1**, 261-267.

Ingham, P. W. and Hidalgo, A. (1993). Regulation of *wingless* transcription in the *Drosophila* embryo. *Development* **117**, 283-291.

Jue, S. F., Bradley, R. S., Rudnicki, J. A., Varmus, H. E. and Brown, A. M. C. (1992). The mouse *Wnt*-1 gene can act via a paracrine mechanism in transformation of mammary epithelial cells. *Mol. Cell. Biol.* **12**, 321-328.

Katzen, A. L., Montarras, D., Jackson, J., Paulson, R. F., Kornberg, T., and Bishop, J. M. (1991). A gene related to the proto-oncogene *fps/fes* is expressed at diverse times during the life cycle of *Drosophila melanogaster*. *Mol. Cell. Biol.* **11**, 226-230.

Kirschmeier, P. T., Housey, G. M., Johnson, M. D., Perkins, A. S. and Weinstein, I. B. (1988). Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. *DNA* **7**, 219-225.

Kitajewski, J., Mason, J. O. and Varmus, H. E. (1992). Interaction of Wnt-1 proteins with the binding protein BiP. *Mol. Cell. Biol.* **12**, 784-790.

Klingensmith, J., Noll, E. and Perrimon, N. (1989). The segment polarity phenotype of *Drosophila* involves differential tendencies toward transformation and cell death. *Dev. Biol.* **134**, 130-145.

Markowitz, D., Goff, S. and Bank, A. (1988a). Construction and use of a safe and efficient amphotropic retroviral packaging line. *Virology* **167**, 400-406.

Markowitz, D., Goff, S. and Bank, A. (1988b). A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* **62**, 1120-1124.

Martinez Arias, A., Baker, N. and Ingham, P. (1988). Role of the segment polarity genes in the definition and maintenance of cell states in the Drosophila embryo. *Development* **103**, 157-170.

Mason, J. O., Kitajewski, J. and Varmus, H. E. (1992). Mutational analysis of mouse Wnt-1 identifies two temperature-sensitive alleles and

attributes of Wnt-1 protein essential for transformation of a mammary cell line. *Mol. Cell. Biol.* **12**, 521-533.

McMahon, A. P. (1992). The *Wnt* family of developmental regulators. *Trends Genet.* **8**, 236-242.

McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of *Wnt*-1-/*Wnt*-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581-595.

McMahon, A. P. and Bradley, A. (1990). The *Wnt*-1 (*int*-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

Molven, A., Njolstad, P. R. and Fjose, A. (1991). Genomic structure and restricted neural expresion of the zebrafish *wnt*-1 (*int*-1) gene. *EMBO J.* **10**, 799-807.

Moon, R. T. (1993). In pursuit of the functions of the Wnt family of developmental regulators: insights from Xenopus laevis. *Bioessays* **15**, 91-97.

Morata, G. and Lawrence, P. A. (1977). The development of *wingless*, a homeotic mutation of *Drosophila*. *Dev. Biol.* **56**, 227-240.

Noordermeer, J., Meijlink, F., Verrijzer, P., Rijsewijk, F. and Destree, O. (1989). Isolation of the *Xenopus* homolog of *int*-1/*wingless* and expression during neurula stages of early development. *Nucl. Acids Res.* **17**, 11-17.

Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K. and Varmus, H. E. (1984). Mode of proviral activation of a putative mammary oncogene (*int-1*) on mouse chromosome 15. *Nature* **307**, 131-136.

Nusse, R. and Varmus, H. E. (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.

Nusse, R. and Varmus, H. E. (1992). *Wnt* genes. *Cell* **69**, 1073-1087.

Nusslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795-801.

Papkoff, J., Brown, A. M. C. and Varmus, H. E. (1987). The *int*-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway. *Mol. Cell Biol.* **7**, 3978-3984.

Papkoff, J. (1989). Inducible overexpression and secretion of *int*-1 protein. *Mol. Cell. Biol.* **9**, 3377-3384.

Papkoff, J. and Schryver, B. (1990). Secreted *int*-1 protein is associated with the cell surface. *Mol. Cell Biol.* **10**, 2723-2730.

Peifer, M. and Bejsovec, A. (1992). Knowing your neighbors: cell interactions determine intrasegmental patterning in Drosophila. *Trends Genet.* **8**, 243-249.

Perrimon, N. and Mahowald, A. P. (1987). Multiple functions of segment polarity genes in *Drosophila*. *Dev. Biol.* **119**, 587-600.

Pulido, D., Campuzano, S., Koda, T., Modolell, J., Barbacid, M. (1992). D*trk*, a *Drosophila* gene related to the *trk* family of neurotrophin receptors, encodes a novel class of neural cell adhesion molecule. *EMBO J.* **11**, 391-404.

Riggleman, B., Schedl, P. and Wieschaus, E. (1990). Spatial expression of the Drosophila segment polarity gene *armadillo* is posttranscriptionally regulated by *wingless*. *Cell* **63**, 549-560.

Rijsewijk, F., Schuerman, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. (1987a). The *Drosophila* homolog of the mouse mammary oncogene *int*-1 is identical to the segment polarity gene *wingless*. *Cell* **50**, 649-657.

Rijsewijk, F., van Deemter, L., Wagenaar, E., Sonnenburg, A. and Nusse, R. (1987b). Transfection of the *int*-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. *EMBO J.* **6**, 127-131.

Russell, J., Gennissen, A., and Nusse, R. (1992). Isolation and expression of two novel *Wnt*/*wingless* gene homologues in *Drosophila*. *Development* **115**, 475-485.

Shackleford, G. M. and Varmus, H. E. (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.

Shilo, B.-Z. (1987). Proto-oncogenes in *Drosophila melanogaster*. *Trends Genet.* **3**, 69-73.

Sidow, A. (1992). Diversification of the *Wnt* gene family on the ancestral lineage of vertebrates. *Proc. Natl. Acad. Sci. USA* **89**, 5098-5102.

Skaer, H. and Martinez Arias, A. (1992). The *wingless* product is required for cell proliferation in the Malpighian tubule anlage of *Drosophila melanogaster*. *Development* **116**, 745-754.

Struhl, G. and Basler, K. (1993). Organizing activity of *wingless* protein in Drosophila. *Cell* **72**, 527-540.

Thomas, K. R., Musci, T. S., Neumann, P. E. and Capecchi, M. R. (1991). *Swaying* is a mutant allele of the proto-oncogene *Wnt*-1. *Cell* **67**, 969-976.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murine *int*-1 proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847-850.

Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. and Varmus, H. E. (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.

Vaidya, A. K., Lasfargues, E. Y., Sheffield, J. B. and Coutinho, W. G. (1978). Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. *Virology* **90**, 12-22.

van den Heuvel, M., Nusse, R., Johnston, P. and Lawrence, P. A. (1989). Distribution of the *wingless* gene product in *Drosophila* embryos; a protein involved in cell-cell communication. *Cell* **59**, 739-749.

van Lohuizen, M., Frasch, M., Wientjiens, E. and Berns, A. (1991). Sequence similarity between the mammalian *bm1*-1 proto-oncogene and the *Drosophila* regulatory genes *Psc* and *Su*(z)2. *Nature* **353**, 353-355.

van Ooyen, A., Kwee, V. and Nusse, R. (1985). The nucleotide sequence of the human *int*-1 mammary oncogene; evolutionary conservation of coding and non-coding sequences. *EMBO J.* **4**, 2905-2909.

Wieschaus, E. and Riggleman, R. (1987). Autonomous requirement for the segment polarity gene *armadillo* during *Drosophila* embryogenesis. *Cell* **49**, 177-184.

Wilkinson, D. G., Bailes, J. A. and McMahon, A. P. (1987). Expression of the proto-oncogene *int*-1 is limited to specific neural cells in the developing mouse embryo. *Cell* **50**, 79-88.