

Apical Localization of *wingless* Transcripts Is Required for Wingless Signaling

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Summary

Many developing and adult tissues are comprised of polarized epithelia. Proteins that are asymmetrically distributed in these cells are thought to be localized by protein trafficking. Here we show that the distribution and function of the signaling protein Wingless is predetermined by the subcellular localization of its mRNA. High-resolution in situ hybridization reveals apical transcript localization in the majority of tissues examined. This localization is mediated by two independently acting elements in the 3' UTR. Replacement of these elements with non- or basolaterally localizing elements yields proteins with altered intracellular and extracellular distributions and reduced signaling activities. This novel aspect of the *wingless* signaling pathway is conserved and may prove to be a mechanism used commonly for establishing epithelial cell polarity.

Introduction

The majority of cells, including those of most single-cell organisms, are at least transiently asymmetrical in shape, composition, and structure. Epithelial cells, for example, have well-defined apical and basal membranes specialized for the import and export of different molecules. One of the major contributing processes in defining this polarity is the subcellular trafficking of proteins to different membranes or compartments (reviewed in Simons and Ikonen, 1997; Mostov et al., 2000). For transmembrane and secreted proteins, evidence accumulated to date suggests that the majority of this trafficking occurs after protein synthesis via the directed movement of specialized vesicles (Simons and Ikonen, 1997; Ikonen and Simons, 1998; Yeaman et al., 1999).

Another process that may contribute significantly to protein trafficking and cell polarity is the subcellular localization of transcripts prior to translation. Documented accounts of localized transcripts now number well over one hundred, and cover a large range of organ-

isms and cell types (reviewed in Bashirullah et al., 1998). These transcripts also encode a variety of proteins, ranging from transcription factors to components of the cytoskeleton. Although relatively few localized transcripts have been tested for functional relevance, localization has been shown to be important for function in the majority of those cases tested. Some well-characterized examples include *Actin* localization at the leading edge of migrating fibroblasts (Lawrence and Singer, 1986; Kislauskis et al., 1997), *oskar* localization at the posterior pole of *Drosophila* oocytes (Ephrussi and Lehmann, 1992), *Prospero* localization in dividing *Drosophila* neuroblasts (Broadus et al., 1998), and *Ash1* localization in dividing yeast cells (Long et al., 1997; Takizawa et al., 1997).

The transcript under investigation in this study encodes a secreted signaling molecule. To date, there are relatively few examples of localized transcripts that encode signaling molecules and these occur predominantly in oocytes. One example with well-documented functional relevance is the localization of *gurken* (*grk*) transcripts. *grk* encodes a TGF β protein, and localized translation within the *Drosophila* oocyte results in directed secretion to nearby follicle cells (Neuman-Silberberg and Schupbach, 1993). Although there are examples of signal and receptor molecules that are encoded by localized transcripts in epithelial cells (*sevenless* for example; Tomlinson et al., 1987), it has yet to be determined whether or not these transcript localization events have functional significance.

The subject of this study, *wingless* (*wg*), is the prototypical member of the highly conserved *Wnt* gene family. *Wnt* genes encode secreted glycoproteins that serve as major signaling molecules in a large number of embryonic patterning processes (Wodarz and Nusse, 1998). Wingless protein (WG), and its vertebrate ortholog WNT1, function interchangeably in a variety of assays including the oncogenic transformation of mammary cells (Bocchinfuso et al., 1999). This interchangeability is due largely to the striking conservation of downstream components in the signaling pathway (reviewed in Wodarz and Nusse, 1998). Important to this study, the majority of these conserved signaling pathway components are localized or enriched within the apical half of expressing cells. For example, the WG transmembrane receptor, Frizzled (FZ), is enriched at the apical surface of imaginal disc cells (Park et al., 1994). The β -catenin homolog Armadillo (ARM), which translocates the signal to the nucleus, doubles as a structural component of the apically localized adherens junctions (Orsulic and Peifer, 1996). Disheveled (DSH) is enriched apically (Yanagawa et al., 1995; Torres and Nelson, 2000), and components of the ARM-modifying complex, consisting of E-Adenomatous polyposis coli (APC), Shaggy (SHG)/Zeste-White 3 (ZW3), and Axin, are found at or near the apicolateral junctions (McCartney et al., 1999; Yu and Bienz, 1999; Yu et al., 1999). WG itself is apically enriched in and around WG-expressing cells (Gonzalez et al., 1991; Strigini and Cohen, 2000).

Here, we report that, as with many components of the

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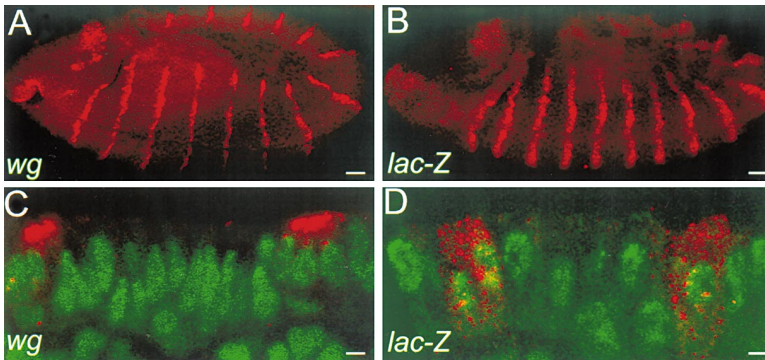


Figure 1. Apical Localization of *wg* Transcripts Is Controlled by the 3' UTR

(A) and (C) show fluorescent in situ hybridization detection of endogenous *wg* mRNA captured by confocal microscopy of stage 8 embryos. (B) and (D) show corresponding expression patterns of transcripts encoded by a *wg-lacZ* enhancer trap line. (A) and (B) are low magnification surface views and (C) and (D) are higher magnification optical cross-sections through the ectodermal layer. Nuclei are green and transcripts red. Scale bars indicate 25 μm in (A) and (B) and 5 μm in (C) and (D).

wg signaling pathway, transcripts encoded by the *wg* gene are also localized apically. This localization is mediated by discrete elements within the *wg* 3' UTR. Redistribution of transcripts within the cell using heterologous 3' UTRs results in a redistribution of the protein and a dramatic loss of signaling activity. The subcellular localization of *Wnt* transcripts in other organisms suggests that this is a functionally conserved aspect of the WNT signaling pathway. Indeed, this mechanism is likely to play a general role in the differential distribution of signaling and transmembrane proteins in other polarized epithelia.

Results

wg Transcripts Are Localized Apically within Cells of the Embryonic Ectoderm

Previous in situ hybridization studies with radioactive probes suggested that *wg* transcripts were enriched apically (Baker, 1987, 1988). Here, we use a recently developed fluorescent in situ hybridization technique (Hughes et al., 1996; Hughes and Krause, 1999) and confocal microscopy to determine precisely the subcellular distribution of *wg* transcripts.

Figure 1 shows the localization of *wg* transcripts in the ectoderm of a mid-stage 8 embryo (Figures 1A and 1C; mRNA: red; nuclei: green). Transcripts encoded by a *lacZ* reporter gene, expressed in the same cells under control of the *wg* promoter, are shown for comparison (Figures 1B and 1D). Optical sections through individual stripes (Figures 1C and 1D) show that differences in the apparent width of each stripe are due to a difference in subcellular transcript localization. Transcripts encoded by the endogenous *wg* gene are confined to a small area just below the apical cell membrane (Figure 1C). In contrast, *lacZ* transcripts expressed in the same cells are distributed uniformly throughout the cytoplasm (Figure 1D). We conclude that this localization is a transcript-specific and not a cell-specific property. Indeed, apical localization of *wg* transcripts is also observed in most other polarized cells (localization in salivary gland cells is sometimes random; data not shown).

Different portions of the *wg* transcript were tested for their ability to confer apical localization in vivo to a nonlocalized *lacZ* transcript (Figure 2A). Transgenic constructs with the *wg* 5' UTR and/or the *wg* ORF, fused either 5' (in-frame) or 3' to the *lacZ* sequence, yielded uniformly distributed transcripts (Figure 2A). However,

fusion of the *wg* 3' UTR to *lacZ* resulted in apical transcript localization (Figure 2A). Thus, the *wg* 3' UTR is both necessary and sufficient for apical transcript localization.

To map the specific sequences responsible for apical localization within the 1098 nt *wg* 3' UTR, deletions were introduced into the *lacZ-wg* 3' UTR reporter, and the deleted reporters tested for their ability to confer apical transcript localization in transgenic embryos (Figure 2B). These deletions defined two *wg* localization elements (WLEs), each of which is sufficient to confer apical transcript localization. WLE1 is located between nucleotides 60–178 and WLE2 is located between nucleotides 670–780 (Figure 2B). These elements may function differently as localization conferred by WLE2 is more closely associated with the apical cortex than that conferred by WLE1 (Figures 2C–2E). Differences in function are also suggested by the lack of apparent similarity in sequence or predicted secondary structure (not shown).

Altering the Subcellular Distribution of *wg* Transcripts

To examine the effect of transcript localization on *WG* signaling, constructs expressing *wg* transcripts that localize to different parts of the cell were made. The three constructs made differ only in their 3' UTRs (Figures 3A–3C). The first uses the endogenous *wg* 3' UTR, the second a 3' UTR derived from the SV40 small t antigen gene, and the third a 3' UTR derived from the *partner of paired (ppa)* gene (Raj et al., 2000). Each of the transgenes was placed under the control of a GAL4-dependent promoter (Brand and Perrimon, 1993) and the vectors introduced into embryos to obtain transgenic flies. Panels D–F in Figure 3 show the transcript distribution patterns obtained when each of these transcripts is expressed under control of a *wg*-GAL4 driver in a *wg* mutant background. As shown above, transcripts containing the *wg* 3' UTR are localized apically (Figure 3D) while transcripts containing the SV40 3' UTR are uniformly distributed (Figure 3E). In contrast, transcripts containing the *ppa* 3' UTR are localized basally (Figure 3F). Note that the distribution of the *ppa*-tagged transcript is not as tightly localized to the basal side of the cell as full-length *wg* transcripts are to the apical surface. Rather, the two distributions appear to be complementary.

Prior to comparing the signaling activities of the proteins made from these three transgenes, Western blot

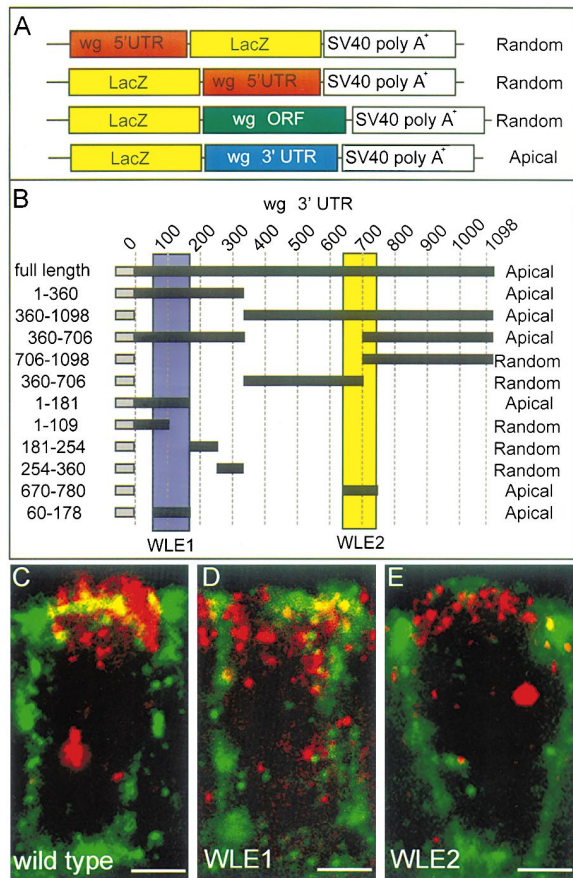


Figure 2. Two Regions within the *wg* 3' UTR Are Sufficient for Apical Localization

(A) *lacZ* fusion constructs containing *wg* transcript 5', ORF, and 3' regions are depicted. Transgenic constructs were expressed in embryos under the control of a *ptc*-GAL4 driver. The cytoplasmic localization of each transcript in ectodermal cells is indicated on the right.

(B) Deletion constructs containing different portions of the *wg* 3' UTR are shown. Transgenic constructs were fused to the *wg* ORF and expressed under *ptc*-GAL4/UAS control. Solid black bars indicate regions remaining. Two minimal localization elements, termed WLE1 and WLE2, are indicated at the bottom. (C), (D), and (E) show subcellular localization of the full-length (C), WLE1-containing (D), and WLE2-containing (E) transcripts depicted in (B). Transcripts are red and cell outlines (anti-phosphotyrosine) are green. The intense medial spots in (C) and (E) are sites of nascent transcription in the nucleus. (Bar = 10 μ m.)

analysis (Figures 4A and 4B) was used to select transgenic lines that express equivalent levels of protein. Expression of the transgenes was induced by crossing the UAS-*wg* flies to *ptc*-GAL4 flies. These express GAL4 in the majority of ectodermal cells. Two matched sets of transgenic lines were selected, a "low"-expressing set and a "high"-expressing set. Quantitation of the protein levels expressed by each of the fly lines in these sets (Figures 4C and 4D) shows that, when the endogenous WG contribution is subtracted, the high lines express about six times the levels of the low lines. Based on the spatial differences between endogenous *wg* and *ptc*-GAL4-driven *wg* expression patterns, we estimate that the high lines express about half the levels of endogenous WG protein on a per cell basis.

Semi-quantitative RT-PCR analysis of the transgenic transcripts shows that transcription levels are also equivalent for each of the three lines in each of the matched sets (Figures 4E and 4F). Initial levels of protein and RNA were also observed to be approximately equal when visualized in situ by immunocytochemistry and in situ hybridization (data not shown). We conclude that the 3' UTR swaps have little effect on the synthesis and stability of *wg* transcripts and protein. Posttranslational modifications also appear to be the same for each protein, as each lane on the Western blot contains a similar set of bands equivalent in number, mobility, and relative intensity (Figures 4A and 4B and data not shown).

The *wg* sequences in each of our constructs, both coding and noncoding, were sequenced prior to injection. Each of the transgenes of the two matched sets was also recovered by PCR and their sequences reconfirmed. Therefore, any differences in protein activity that might be observed amongst matched lines must be attributed to differences in transcript localization.

WG Autoregulation Requires Apical Transcript Localization

WG facilitates its own expression via both autocrine and paracrine signaling pathways (Hooper, 1994; Manoukian et al., 1995; Yoffe et al., 1995). To test whether the localization of *wg* transcripts affects these activities, pulses of *wg* construct expression were induced by crossing the high set of UAS-*wg* transgenic flies to a heat shock-Gal4 line and subjecting 3- to 5-hr-old embryos to a 30 min heat shock. Protein levels were assessed by Western blot analysis (Figures 5A and 5B).

Immediately following the heat pulse, each of the matched transgenic lines produced the same amount of protein. This was about three times the amount of endogenous WG expressed in heat-shocked controls. In the apical transcript line, these levels rose about 5-fold higher during the next half hour, and subsequently remained at a high level. This increase in expression levels is due to the spatial expansion and intensification of endogenous WG stripes (Noordermeer et al., 1992). In the line with uniform *wg* transcript distribution, autoregulation also occurred but with slower kinetics. In contrast, the basal transcript line showed no further increase in WG expression levels 30 min after the heat pulse, and by 60 min, expression levels were similar to those seen in the heat shock control. Transcript levels for each of the lines and each time point were also measured using RT-PCR and NIH image, and confirmed that, as with the *ptc*-GAL4-driven expression, each of the transgene mRNAs was expressed and turned over at equivalent rates (data not shown). Thus, we conclude that apical transcript localization is important for WG autoregulation.

WG Rescuing Activity Requires Apical Transcript Localization

In order to test the signaling activities of our differentially localized transcripts in a more comprehensive fashion, each of the constructs of our high- and low-expressing matched sets was tested for their ability to rescue *wg*-dependent segmental patterning. This was accomplished by recombining the two sets of *wg*-expressing

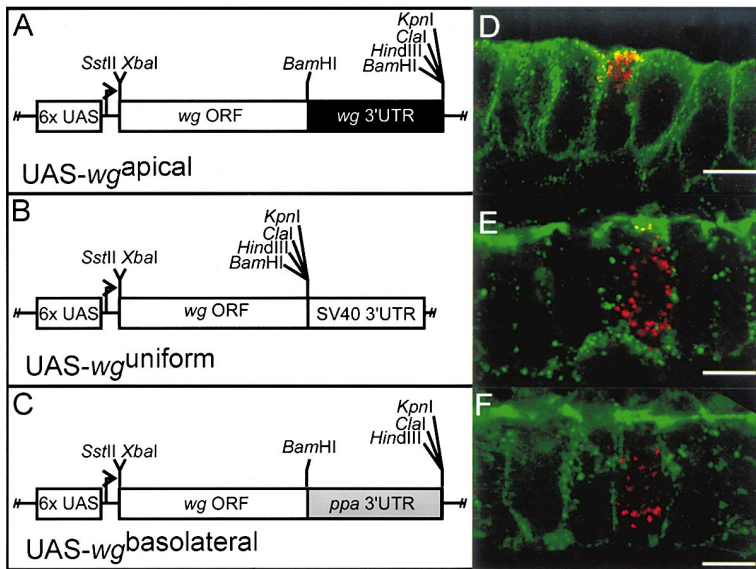


Figure 3. Differential Localization of *wg* Transcripts

wg constructs with different 3' UTRs were made to localize transcripts to different regions of the cytoplasm. All three constructs express *wg* under the control of a GAL4-dependent promoter. The construct in (A) contains the full-length *wg* 3' UTR. The construct in (B) has the *wg* 3' UTR replaced by the SV40 small t antigen gene 3' UTR. The construct in (C) contains the 3' UTR of the *partner of paired* gene in place of the *wg* 3' UTR. (D)–(F) show the subcellular distributions of the transcripts encoded by each transgene when expressed in *wg*-expressing cells in a *wg* mutant background. Transcripts are shown in red and cell outlines (α -spectrin or anti-phosphotyrosine) in green. (Bar = 10 μ m.)

transgenic lines into a *wg* null mutant background and crossing these lines to flies that express GAL4 under *wg* promoter control (Hays et al., 1997). As expected, the apically localized transcript of the high apical line is capable of restoring much of the naked cuticle that is missing in *wg* mutant embryos (Figure 6B). The incomplete nature of this rescue is most likely due to sub-optimal levels of expression as compared to the endogenous *wg* gene (about 50%; Figures 4 and 7). In comparison, the high uniform construct yields significantly reduced rescuing activity (Figure 6C) and the high basolateral line very little rescuing activity (Figure 6D). The low set of lines shows a similar trend, but with substantially lower degrees of rescue (Figures 6E and 6G). Note that the low apical line yields more rescuing activity than the high basal line despite expressing only 1/6th the levels of protein. Similar relative activities were observed using other GAL4 drivers and with other constructs that were not part of the matched sets (data not shown). We conclude that transcript localization within apical cytoplasm is essential for robust signaling activity.

WG Protein Distribution Is Affected by Transcript Localization

In order to help understand how transcript localization affects protein function, we looked to see if differences in protein distribution, in and around *wg*-expressing cells, could be detected. Expression of the three transgenes in the high matched set was driven using a *wg*-GAL4 driver, and WG distributions were observed in a *wg* null background. The single-cell-wide *wg* stripes (Figures 3D–3F) serve as a point source from which diffusion of the protein, laterally and apically/basally, can be readily observed.

Using confocal microscopy, most of the WG protein detected in wild-type embryos is enriched in the apical cytoplasm of *wg*-expressing cells (Figures 7A and 7B). Although the majority of this signal is diffuse, brightly staining punctate bodies are also observed. Similar punc-

tae are also found in cells nearby (Strigini and Cohen, 1999; Pfeiffer et al., 2000; Figures 7A and 7B). In the *wg*-expressing cells, these punctate bodies are thought to represent both endocytic (Gonzalez et al., 1991) and exocytic vesicles (Pfeiffer et al., 2000).

WG expressed from the apically localized transgene transcript (Figures 7C and 7D) is distributed much the same as in the wild-type control. However, protein expressed from the uniformly distributed transcript (Figures 7E and 7F) shows clear differences in distribution. Although it still appears to be somewhat enriched in the apical cytoplasm of *wg*-expressing cells, there is less of the protein in these cells and more extending laterally into the middle of the segment. The difference in distribution of protein translated from the basal transcript is even more striking (Figures 7G and 7H). There is little detectable enrichment within the *wg*-expressing cells, and more of the protein extends laterally across the segment. Interestingly, this extracellular protein still appears to be apically enriched. We conclude that protein synthesized basally is secreted more efficiently, diffuses more rapidly within the extracellular matrix, or is less effectively endocytosed.

Discussion

Subcellular Localization of *wg* Transcripts Correlates with WG Signaling Activity

One of the paradigms of cell biology is that proteins integrated within or secreted from a particular cell surface are generally synthesized from uniformly localized transcripts. These transcripts bind to rough endoplasmic reticulum near the nuclear perimeter, and sorting begins after the protein-containing vesicles leave the Golgi stacks. Here, we show that the distribution of a secreted protein can also be controlled at the level of transcript localization.

With the aid of high-resolution in situ hybridization, we have shown that *wg* transcripts are enriched within the apical cytoplasm of most epithelial cells examined.

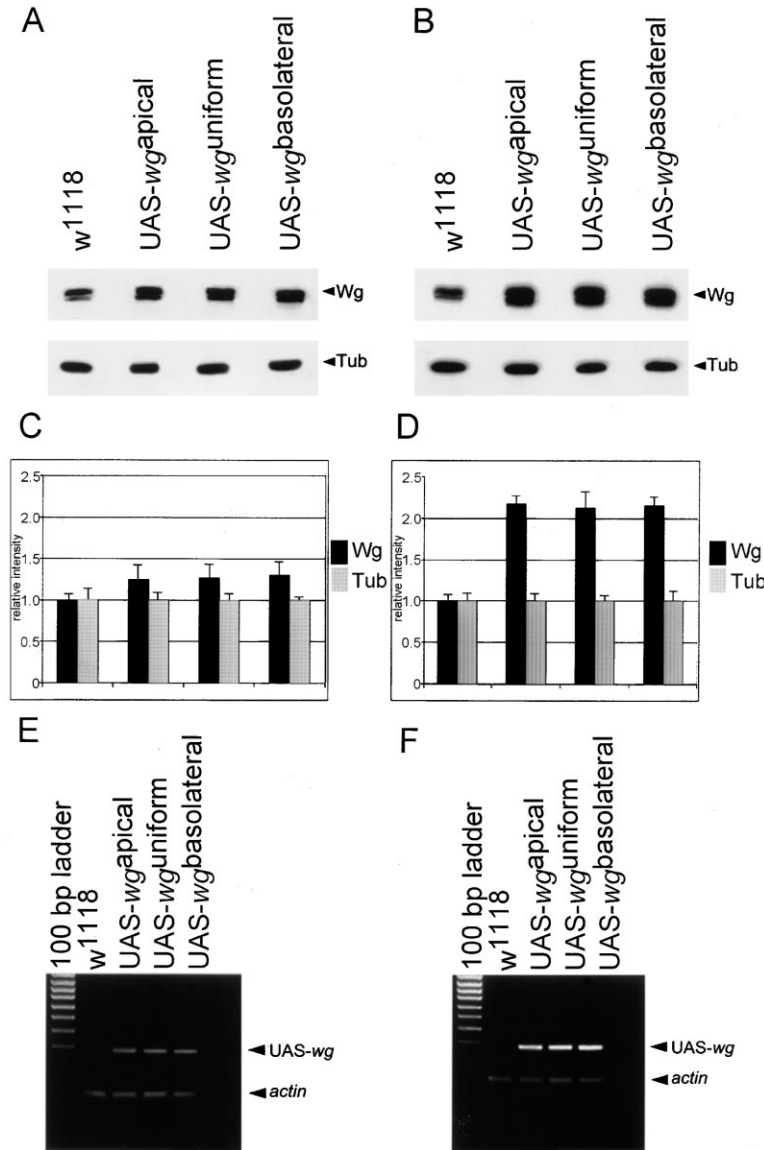


Figure 4. Selection of Matched WG-Expressing Transgenic Lines

(A) and (B) show representative Western blots of WG protein expressed from matched apical, uniform, and basal *wg*-expressing transgenic lines. (A) shows protein expressed from a low-expressing set of lines, and (B) shows protein expressed from a high-expressing set of lines. Tubulin loading controls performed on the same blots are shown below. (C) and (D) are graphs showing phosphorimager-derived values for the relative levels of WG expression in each of the transgenic lines as compared to endogenous levels (normalized to tubulin). Black bars are WG expression levels and hatched bars are tubulin expression levels. Error bars indicate standard deviations for four low set and ten high set blots. (E) and (F) show mRNA expression levels as detected by RT-PCR. The agarose gel in (E) reflects mRNA levels expressed by the low set of matched lines and the gel in (F) reflects mRNA levels expressed by the high set. Transcripts encoded by the actin gene were also amplified as an internal control.

Indeed, the majority of this mRNA is concentrated just below the apical cell surface, well away from the nuclear envelope. When these transcripts are redistributed to the basal cytoplasm, the protein is efficiently translated but shows little signaling activity. This loss in activity correlates with changes in protein distribution. Less of the protein is found within the apical cytoplasm of *wg*-expressing cells and more is found in or around the cells nearby. Preliminary expression studies at other stages and in other tissues suggest that apical transcript localization is a general requirement for *wg* signaling in epithelial cells.

Potential Reasons for Transcript Localization

By controlling where in the cell transcripts are translated, localization could potentially affect a number of protein properties. In the case of a secreted protein such as WG, transcript localization might control the organelle, or subregion thereof, where the protein would ultimately be synthesized. This, in turn, may affect trans-

lation efficiency, cleavage, posttranslational modifications, cofactor association, or trafficking of the newly synthesized protein.

If localized translation of *wg* transcripts is affecting processing, these effects must be very subtle. Our Western blots revealed no obvious differences in translation efficiency, molecular weight, or glycosylation. Thus, apically and basally translated proteins are entering the secretory pathway with similar efficiencies. This rules out the possibility of endoplasmic reticulum (ER) or golgi apparatus restriction to different parts of the cell. It does not, however, rule out the possibility of differential compartmentalization along the apical/basal axis. For example, if Porcupine (PORC), an ER protein required for WG exocytosis (van den Heuvel et al., 1993), were enriched within an apical subregion of the ER, then protein synthesized basolaterally might be processed differently and/or enter a different secretory pathway together with different cofactors. Either possibility could affect protein secretion, diffusion, or receptor binding. Recent support

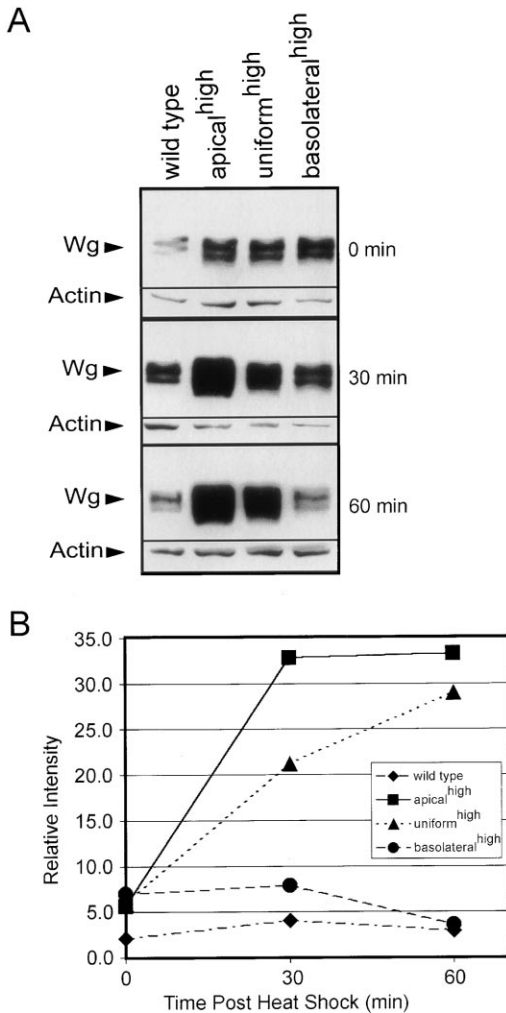


Figure 5. Autocrine *wg* Signaling Requires Apical Transcript Localization

(A) shows Western blots of Wg expressed in heat shocked wild-type and HS-GAL4/UAS-*wg* transgenic constructs following a 30 min heat shock. Protein was extracted from whole embryos at 0, 30, and 60 min post heat shock. Actin loading controls are shown below each corresponding Wg signal. (B) shows a plot of the relative levels of protein detected in the blots above. Levels are corrected for loading and are relative to Wg expression levels in the heat shocked control at 0 min. The signals shown were all within the linear range of phosphoimager detection.

for the possibility of different ER or golgi compartments comes from the observation that yeast GPI-linked proteins are shuttled within a specific subset of vesicles between these two organelles (Muñiz et al., 2001).

Once Wg is secreted, interactions with heparin sulfate-containing proteoglycans (HSPGs) within the extracellular matrix are required for proper signaling (Bradley and Brown, 1990; Jue et al., 1992; Reichsman et al., 1996; Hacker et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999). These HSPGs are thought to function as low affinity receptors that act at one of two levels, either to facilitate binding to FZ receptors or to limit diffusion away from FZ receptor complexes. These proteins are also required for signaling by other WNT proteins as

well as by members of the Hedgehog, TGF β , and FGF families of signaling molecules (reviewed in Baeg and Perrimon, 2000; Christian, 2000; Selleck, 2000). Differential distribution or modifications of these extracellular matrix proteins along the apical/basal plane might explain the observed differences in Wg diffusion and activity when translated basally. Although apical/basal differences in the distribution of other extracellular matrix proteins are well documented (reviewed in Fessler and Fessler, 1989; Gullberg et al., 1994; Murray et al., 1995), these particular proteins have yet to be analyzed.

Another possible consequence of basal Wg secretion may be the failure to associate effectively with FZ receptor complexes. As with many other receptor complexes, many of the proteins that bind and mediate the Wg signal appear to be closely associated and apically enriched (Woods and Bryant, 1993; Yanagawa et al., 1995; Torres and Nelson, 2000; Orsulic and Peifer, 1996; McCartney et al., 1999; Yu and Bienz, 1999; Yu et al., 1999). Wg may need to be secreted from the apical surface, perhaps even via a specific secretory pathway, in order to interact productively with these functional protein complexes. In imaginal discs, FZ itself is apically localized (Park et al., 1994). FZ2, however, is not (Strigini and Cohen, 2000), indicating that the receptor itself need not be the spatially limiting component.

In most *wg*-expressing tissues, layers of cells are found basal to *wg*-expressing cells, and in some tissues, facing the apical surface as well. Although not addressed here, apical localization may also provide a mechanism to direct signals toward apically or laterally opposed cells and away from basally opposed cells. This possible role as a determinant of signaling direction requires further study.

Conservation of *wg* Transcript Localization

Transcripts encoded by the *Drosophila virilis wg* gene have been examined to see if apical transcript localization is conserved in this species. Despite an estimated evolutionary divergence of about 60 million years, and the tendency of 3' UTRs to diverge rapidly in sequence, this transcript is also localized apically (A. J. S. and H. M. K., unpublished data). Indeed, we find that the *D. virilis wg* 3' UTR is functional in *D. melanogaster*, and elements with sequence similarity to the two *wg* localization elements, WLE1 and WLE2, exist in similar positions within the 3' UTR. Although conserved between species, the sequences and predicted secondary structures of WLE1 and WLE2 bear no resemblance to one another. Their sequences also fail to show significant homology to other sequences in the database, including those of other localized transcripts. Taken together with the observation that *wg* transcripts colocalize to the same particles as other localized transcripts (Wilkie and Davis, 2001 [this issue of *Cell*]), we surmise that transcript recognition is mediated either by transcript-specific adaptors or by common adaptors that recognize similar secondary structures.

This functional conservation of *wg* localization elements in *Drosophila* further substantiates the importance of *wg* transcript localization and suggests that this step in the pathway may be conserved in other organisms. Indeed, a number of vertebrate *wnt* tran-

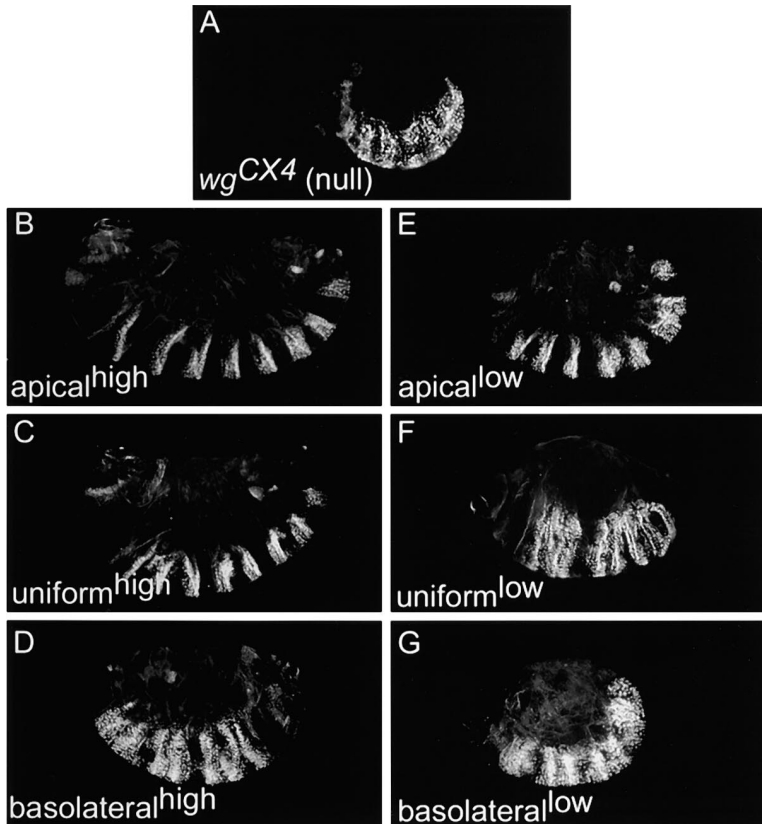


Figure 6. Apical Transcript Localization Is Required for *wg* Rescuing Activity

The UAS-regulated transgenes of our two matched sets were expressed under control of a *wg*-GAL4 driver in a *wg* mutant background, and cuticles were prepared at the end of embryogenesis. (A) shows a *wg*^{CX4} cuticle in which the naked regions between denticle belts are deleted and the length of the cuticle is greatly reduced. (B) and (E) show characteristic rescue mediated by apically localized *wg* transcripts, (C) and (F) by uniformly localized transcripts and, (D) and (G) by basally localized transcripts. Cuticles on the left (B, C, and D) are characteristic for rescue by the high-expressing set of lines and cuticles on the right (E, F, and G) for rescue by the low set of lines. The phenotypes shown are representative of the range produced by each construct (n = 100–200/construct).

scripts have been shown to be localized within oocytes. For example, transcripts encoded by the *Xwnt5* and *Xwnt11* genes of *Xenopus* are localized in the oocyte vegetal pole while those of *X-Wnt8b* are localized to the animal pole (Ku and Melton, 1993; Moon et al., 1993; Cui et al., 1995). In *Ascidians*, the maternally expressed *HrWnt-5* transcript is localized to the posterior of early embryos (Sasakura et al., 1998). Recent advances in in situ hybridization technologies should now permit the detection of polarized transcript distributions in the somatic cells of these and other organisms.

Transcript Localization in Other Signaling Pathways

A number of signaling molecules in addition to WNTs are known to be translated from localized transcripts. These include the *Drosophila* proteins Gurken, Sevenless, Short gastrulation, Twisted gastrulation, and the *Xenopus* protein Vg1 (Banerjee et al., 1987; Weeks and Melton, 1987; Neuman-Silberberg and Schupbach, 1993; Francois et al., 1994; Mason et al., 1994). For some of these that are expressed in the oocyte, transcript localization has been shown to be important (Neuman-Silberberg and Schupbach, 1994). The results presented here show that transcript localization can also regulate signaling protein distribution and activity in polarized epithelia, where it may serve as either an alternative or a supplement to vesicle-mediated protein trafficking. Given the observations that at least 10% of transcripts have the potential to be localized (Dubowy and Macdonald, 1998) and that epithelial cells possess the *trans-*

acting machinery required for localization (this study; Wilkie and Davis, 2001 [this issue of *Cell*]), this mode of signaling molecule regulation may prove to be relatively common. This mode of protein trafficking may also be used for other types of polarity-conferring proteins such as intercellular adhesion and cytoskeleton proteins.

Experimental Procedures

Fly Stocks and P-Element Transformations

In all cases, a *w*¹¹¹⁸ stock was used as a wild-type control. The *w*; *Sb*Δ2–3/TM6 *Ubx*, *wg*^{CX4}, *ptc*-GAL4, *wg*-GAL4, and heat shock-GAL4 (HS-GAL4) lines were obtained from the Bloomington *Drosophila* Stock Centre. All other stocks are described in Lindsley and Zimm (1992).

pUAST (Brand and Perrimon, 1993) derived constructs (see below) were each independently sequenced and then introduced by microinjection (Rubin and Spradling, 1982) into Δ2–3 embryos (Robertson et al., 1988) to generate transgenic fly lines. Ten to twenty independent lines of each UAS-*wg* transgene were generated and all were tested for levels of protein and mRNA expression as described below. For the *wg*-GAL4 rescue experiments, UAS-*wg* transgenes on the second chromosome were recombined with a second chromosome containing a *wg*^{CX4} null allele.

Construction of *wg*/*lacZ* Fusion and *wg* Protein-Expressing Constructs

The *wg* ORF, 5' and 3' UTR sequences were tested for localizing activity by subcloning each component downstream of the *lacZ* coding sequence. The *wg* ORF was excised using a 5' NcoI site and a 3' *Afl*III site located at the beginning of the 3' UTR. The 5' UTR was subcloned using the 5' BamHI and 3' XhoI sites such that the *lacZ* ORF would be in-frame with the *wg* signal peptide. The same fragment was also subcloned 3' to the *lacZ* sequence using a 3' NcoI site. To subclone the *wg* 3' UTR, a 5' SacI site was

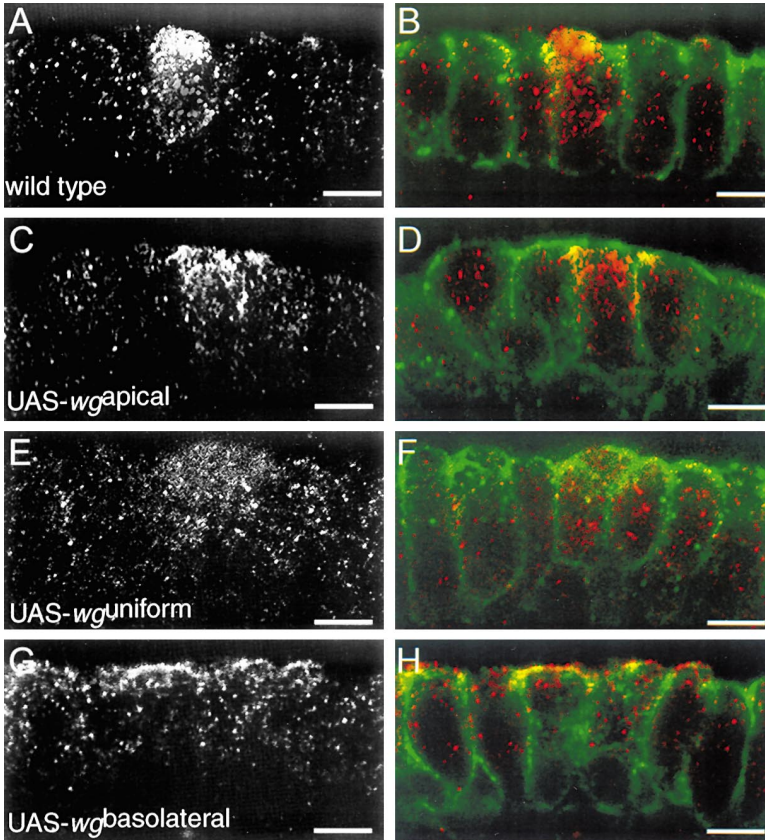


Figure 7. Effects of *wg* Transcript Localization on WG Protein Distribution

Each of the UAS transgenes of our high set was expressed under control of a *wg*-GAL4 driver in a *wg* mutant background. Panels on the left show WG alone and panels on the right show composites of WG (red) and cell outlines (phosphotyrosine; green). (A) and (B) show endogenous WG localization and (C)–(H) the transgenic proteins. (C) and (D) show protein expressed from apical transcripts, (E) and (F) from uniform transcripts, and (G) and (H) from basal transcripts. Each panel represents a single 0.2 μm confocal section. (Bar = 10 μm .)

introduced at the 5' end by PCR using the oligo ACGTGCTCACAGA TACTCGAGGGC (all oligos are written 5'-3') and a 3' plasmid-specific T7 primer. The 3' UTR was then excised with *SacI* and a *BglII* site located approximately 100 base pairs downstream from the predicted end of the cDNA (Nusse et al., 1984).

To facilitate fine mapping of the localization elements in the *wg* 3' UTR, a modular *wg* ORF fragment was first created from the *wg* cDNA. An *XbaI* site was introduced immediately upstream of the start codon and a *BamHI* site 3' to the stop codon using the primers GGTCTAGATGGATATCAGCT and GGGGATCCTTACAGACACGTGA and high-fidelity PCR using *Pfu* polymerase (Stratagene). Similarly, a modular 3' UTR fragment was made by introducing *BamHI* restriction sites at each end of the *wg* 3' UTR using the primers GGGGATC CACACTGCCCGCCT and GGGGATCCACTTGGCTTTTA. Large deletions in the 3' UTR were made using internal *AflIII* (position 109), *ClaI* (position 360), and *HindIII* (position 706) restriction sites. Smaller deletions in the 1–360 construct were introduced using internal *AluI* (position 178) and *BfI* (position 107) sites. The minimal localization elements were generated by PCR using the primers GGGATCCTG TATGTGTTATGT and GGGGATCCAGAGCTTAAAGGGTT for WLE1, and GGGGATCCCAACAAATTCTTTTT and GGGGATCCGCAAC TAAATCTT for WLE2.

The uniformly localized UAS-*wg* construct was created by substituting the *wg* 3' UTR with that of the SV40 small t antigen gene 3' UTR, already present in the pUAST vector (Brand and Perrimon, 1993). The basolateral localizing UAS-*wg* construct was made by fusing the *wg* ORF clone to a *BamHI*-*HindIII* fragment containing the 3' UTR of the *partner of paired* (*ppa*) gene (Raj et al., 2000). For each of the latter three constructs, the coding and 3' UTR sequences were sequenced for errors introduced by cloning prior to injection into *Drosophila* embryos. These sequences were confirmed again after isolation of the transgenic lines using the pUAST-specific primers CCGGAGTATAAATAGAGGCGCTTCG and GTCACACCACAGAA GTAAGTTCC to amplify the transgenic sequences (Gloor et al., 1991). The amplified sequences were sequenced using the internal primers GCTAAGCGAAAGCTAAGC and GATCCTCTAGAGGTAC.

Quantitation of *wg* Transgene Expression Levels

The relative expression of WG within each of the transgenic lines was examined by quantitative Western blotting using both actin and β -tubulin signals as loading controls. For each line, 250 *ptc*-GAL4 virgin females were crossed to 200 males of each UAS-*wg* line. These were allowed to mate for 3 days, and embryo collections made on the fourth and fifth days. For the heat shock time course experiment, HS-GAL4 was used instead of *ptc*-GAL4. Embryos were collected for 2 hr, aged for 3 hr, and then heat shocked for 30 min in a 36.5°C water bath. Total protein was extracted as described below at 0, 30, and 60 min after heat shock. Embryos were first dechorionated in a 50% bleach solution, then flash-frozen in liquid nitrogen and transferred into 200 μL 1 \times SDS-PAGE loading buffer on ice. Embryos were then homogenized using a plastic pestle, and the resulting extract boiled for 5 min. Insoluble material was pelleted using a 1 min microcentrifuge spin and 10 μL of the supernatant loaded onto a 10% SDS polyacrylamide gel. Resolved proteins were transferred to 0.2 μm nitrocellulose membranes (S&S) using standard techniques and the membranes blocked overnight in PBTB (1 \times PBS + 0.2% Tween-20 and 0.2% skim milk powder). The *wg* monoclonal antibody 4D4 (diluted 1:350; Brook et al., 1996), the anti- β -tubulin polyclonal antibody E7 (diluted 1:600; Chu and Klymkowsky, 1989), and the anti-actin monoclonal antibody JLA20 (diluted 1:400; Lin, 1981) were obtained from the Developmental Studies Hybridoma Bank (developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, Iowa). These were diluted in PBTB, incubated for 90 min at room temperature, and then washed six times for 5 min with PBTB. The membrane was then incubated in anti-mouse horseradish peroxidase conjugated secondary antibody (diluted 1:25,000; Pierce) for 90 min and washed six times in PBT (1 \times PBS + 0.2% Tween-20) for a total of 30 min. Signals were developed using Supersignal-West-Dura chemiluminescent substrate (Pierce), and the WG-, tubulin-, and actin-specific bands quantified using a BioRad G360 Phosphoimager. Loadings were varied over a two order of magnitude range to ensure that the signals detected and quantified were in the linear range of the phosphoimager.

To measure transcript levels by RT-PCR, RNA was isolated from 4–6 hr *ptc*-GAL4/UAS-*wg* or HS-GAL4/UAS-*wg* embryos using an RNeasy mini column (Qiagen), followed by a one-step RT protocol (Qiagen), using the following primers: ATCATACCCGGTGTGTCAG TGTGAGA (*wg* 5'), ACCAGCAACCAAGTAAATCAACTGCA (UAS 5'), GGGCGTAATGTTGGGTTCCG (*wg* 3'), AGCCAGCAGTCGTCTAA TCCAG (actin 5'), and CAGCAACTTCTTCGTCACACAT (actin 3'). During RNA extraction, the columns were incubated for 20 min with DNase (Qiagen) before elution to prevent DNA contamination of the RNA templates. RT-PCR was then performed on equal amounts of RNA, as described by the manufacturer. The RT reaction was allowed to proceed at 50°C for 30 min followed by 95°C for 15 min. The subsequent PCR cycling parameters were 40 cycles of: 94°C for 40 s, 55°C for 40 s, and 72°C for 60 s, followed by a final 10 min at 72°C. The resulting products were separated on 2% agarose gels, photographed using a digital camera, and the relative intensities of the bands quantified using NIH-Image for Windows 2000 (Scion).

Immunofluorescence and Fluorescent In Situ Hybridization

In situ hybridization, fluorescent detection of hybridized transcripts, and fluorescent detection of α -Spectrin in whole-mount embryos was performed as described previously (Hughes et al., 1996; Hughes and Krause, 1999). The *wg* RNA probe used encompassed the entire ORF and was Digoxigenin labeled during run-off transcription as per the manufacturer's directions (Roche). Rabbit α -phosphotyrosine or mouse anti-phosphotyrosine (NEB) antibodies were used at a dilution of 1:300 and 1:1000, respectively. Embryos were incubated at 4°C overnight with either mouse anti-WG 4D4 1:10 or affinity-purified rabbit anti-WG 1:50 diluted in PBTB. Embryos were then washed four times at room temperature, 30 min each time, with PBTB. WG antibody was detected using either an Alexa488 conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Molecular Probes), and the resulting signal was further amplified using an Alexa488 conjugated donkey anti-goat antibody, each diluted 1:1000 in PBTB (Molecular Probes). Fluorescent antibodies were added sequentially, each time incubating for 90 min and following with four 20 min washes in PBTB. The anti-phosphotyrosine and anti-phosphotyrosine antibodies were detected using donkey anti-rabbit or donkey anti-mouse Cy5 secondary antibodies added 90 min before the final series of washes (Jackson Immunologicals). Nuclei were counterstained using propidium iodide (1 μ g/ml; Sigma Aldrich, Inc) added 5 min prior to the final washes. Images were obtained using a Leica TCS NT laser-scanning confocal microscope. Each fluorochrome was scanned individually to avoid bleed-through between channels. Images were subsequently combined using Adobe Photoshop 5.1.

Cuticle Preparations

Embryos were collected and cuticles prepared as previously described (Saulier-Le Drean et al., 1998). Approximately 400–500 cuticles were scored for each cross.

Acknowledgments

We wish to thank H. Lipshitz, C. Smibert, I. Davis, U. Tepass, and various members of the Krause laboratory for comments on the manuscript. We also thank R. Nusse for *wg* cDNA, M. Weir for *ppa* cDNA, D. Branton for rabbit α -Spectrin, and S. Cohen and K. Cadigan for α -WG antibodies. This work was supported by a grant from the National Cancer Institute of Canada. A. J. S. is supported by a K.M. Hunter Fellowship in Cancer Research from the NCIC and G. dS. by a Medical Research Council of Canada studentship.

Received December 1, 2000; revised February 21, 2001.

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