The *Drosophila* tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation

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Homozygous loss of the warts (wts) gene of Drosophila, caused by mitotic recombination in somatic cells, leads to the formation of cell clones that are fragmented, rounded, and greatly overgrown compared with normal controls. Therefore, the gene is required for the control of the amount and direction of cell proliferation as well as for normal morphogenesis. The absence of wts function also results in apical hypertrophy of imaginal disc epithelial cells. Secretion of cuticle over and between the domed apical surfaces of these cells leads to a honeycomb-like structure and gives the superficial wart-like phenotype of mitotic clones on the adult. One wts allele allows survival of homozygotes to the late larval stage, and these larvae show extensive imaginal disc overgrowth. Because of the excess growth and abnormalities of differentiation that follow homozygous loss, we consider wts to be a tumor suppressor gene. The wts gene is defined by the breakpoints of overlapping deficiencies in the right telomeric region of chromosome 3, region 100A, and by lethal P-element insertions and excisions. It encodes a protein kinase that is most similar to human myotonic dystrophy kinase, the Neurospora cot-1 protein kinase, two cell-cycle regulated kinases of yeast, and several putative kinases from plants. These proteins define a new subfamily of protein kinases that are closely related to but distinct from the cyclic AMP-dependent kinases. Although myotonic dystrophy is defined by a neuromuscular disorder, it is sometimes associated with multiple pilomatrixomas, which are otherwise rare epithelial tumors, and with other tumors including neurofibromas and parathyroid adenomas. Our results raise the possibility that homozygous loss of the myotonic dystrophy kinase may contribute to the development of these tumors.

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The origin and progression of human tumors involves a series of genetic changes including the activation of oncogenes and the loss or inactivation of tumor suppressor genes (Schmandt and Mills 1993). The importance of tumor suppressor genes is now well recognized with the molecular identification of many of them and the demonstration of normal allele loss in many types of tumors (Knudson 1993). The products of tumor suppressor genes include cell adhesion proteins, signal transduction molecules, transcription factors, and molecules involved in the control of the cell cycle and of apoptotic cell death (Knudson 1993). The use of animal model systems with easily manipulable genetics provides an approach to the rapid identification and characterization of tumor suppressor genes. Mutagenesis screens and the identification of spontaneously occurring mutations in *Drosophila* have already led to the identification and genetic mapping of >50genes in which mutations give an overgrowth phenotype, and therefore, that are considered tumor suppressor genes (Watson et al. 1994). Eight of these function in the embryo, 12 in the developing brain, 19 in the developing imaginal discs, 25 in the developing hematopoietic system, and 10 in the adult gonads. Some of these genes function in more than one of these locations.

³This work resulted from an equal contribution by these authors.

Of the 22 Drosophila tumor suppressor genes that

have been cloned and characterized at the molecular level, 4 function in imaginal discs. These four show clear homology to human genes, none of which had been recognized previously as a tumor suppressor gene. Thus, the fat gene encodes an enormous cadherin-like transmembrane molecule that is probably involved in cell adhesion (Mahoney et al. 1991). The discs-large (dlg) gene encodes a protein containing an SH3 domain and a region with guanylate kinase homology that is associated with septate junctions (Woods and Bryant 1991); it shows homology to mammalian proteins in tight junctions, synaptic junctions, and the erythrocyte membrane cytoskeleton (Bryant and Woods 1992; Woods and Bryant 1993). The protein product of lethal(2)giant larvae (lgl) is found either in the cytoplasm or in association with cell membranes at sites of cell-cell contact (Strand et al. 1994), and has a closely related mammalian homolog that is regulated by Hox-C8 but is otherwise of unknown function (Tomotsune et al. 1993). expanded (exp) shows homology to the mammalian NF2 tumor suppressor gene (Boedigheimer et al. 1993), which encodes a member of the protein 4.1 family of membrane-cytoskeletal linker proteins. The fact that these four genes all encode membrane-associated proteins supports the idea, derived mainly from regeneration experiments (Bryant 1987), that cell proliferation is controlled by contact-dependent interactions between cells and their neighbors in these growing epithelial tissues.

The effects of homozygous loss of tumor suppressor genes can be assessed easily in Drosophila by inducing mitotic recombination in a developing fly that is heterozygous for a deficiency or mutant allele of the gene. Mitotic recombination can be induced at random chromosome sites by irradiation (Becker 1974), or at specific sites by using a yeast recombination system that has been introduced into Drosophila by genetic transformation (Golic and Lindquist 1989). In the case of fat (Mahoney et al. 1991), loss of both wild-type alleles in mitotic clones leads to clone overgrowth and the production of differentiated outgrowths on the adult. Simultaneous loss of the lethal(2)giant discs (lgd) gene (Bryant and Schubiger 1971) causes a dramatic increase in the size of these growths, although lgd itself does not cause clone overgrowth (M. Buratovich and P. Bryant, unpubl.). Loss of both normal alleles of the *dlg* gene produces abnormally differentiated outgrowths on the adult (Woods and Bryant 1991). Mitotic recombination clones can also be used to search for new tumor suppressor genes, as the overgrowth of the clone can be detected readily, at least in the cases where adult differentiation is not blocked completely.

Here, we describe a new tumor suppressor gene, warts (wts), identified by its clone phenotype in Drosophila. Loss of the wts gene not only results in overproliferation but also in apical hypertrophy of epithelial cells, leading to abnormal deposition of extracellular matrix (cuticle) during adult development. Thus, the wts gene function is important in controlling cell growth and shape as well as proliferation. The wts gene shows highly significant homology to a gene in Neurospora that is required for the control of cell shape and to the human gene in which mutations cause myotonic dystrophy.

Results

Phenotype of wts deficiencies in mitotic recombination clones

The presence of the wts gene was inferred initially from the observation that mitotic recombination clones homozygous for the deficiency Df(3R)A177der20 produce spectacular outgrowths from the body surface (Fig. 1). These clones were generated in flies that carry a dominant bristle-shape mutation, Kinkeds (Kis; Gausz et al. 1994), on the nondeficiency chromosome. All bristles outside of the Df(3R)A177der20 clone are short and bent because of Ki^s, whereas those inside the clone (and lacking the wts gene as well as the Ki^s mutation) are normal (Fig. 1). The area of the wts clone corresponds to the overgrown area of the adult epithelium, which also has an unusually rough and/or honeycomb-like appearance. The loss of wts gene function, therefore, leads to the cell-autonomous formation of epithelial tumors in the adult integumentary structures derived from imaginal discs.

The wts clones are larger than control clones produced in the same fly. With irradiation (1000 rads) at 3 days after egg laying, Df(3R)A177der20 clones on the adult wing are almost twice as large in area (mean area, $5.7\pm0.4\times10^3$ µm²; n = 154) as multiple wing hair (mwh) control clones (mean area, $3.6 \pm 0.4 \times 10^3 \,\mu\text{m}^2$; n = 67), indicating that the constituent cells undergo more divisions than normal. The Df(3R)A177der20 clones are also more than twice as frequent as control clones in the same experiment (7.6 wts clones/wing vs. 3.3 mwh control clones/wing; n = 20). The high frequency of the deficiency clones as well as the presence of clusters of mutant patches on individual wings and legs (Fig. 1) indicates that single clones split into separate pieces during their growth. If this is true, then the effect of the deficiency on growth within clones is about double what is indicated by simply comparing average clone sizes.

The Df(3R)A177der20 clones have smooth edges and are round or elliptical in shape on legs and especially wings, whereas control clones marked with *mwh* are irregular and elongated on these appendages (Fig. 2). The rounded shape of *wts* clones suggests that the divisions of cells lacking *wts* gene function are not oriented preferentially as they appear to be in wild-type imaginal discs (Bryant and Schneiderman 1969; Bryant 1970; García-Bellido and Merriam 1971), or that there is a defect in cell adhesion that leads to abnormal cell arrangements.

Effects of wts deficiencies and mutations on cell shape

The texture of the cuticle in *wts* clones is very unusual in that cell outlines are clearly visible, producing a rough and/or honeycomb-like appearance, whereas cell boundaries are not visible in surrounding wild-type tissue (see Fig. 1). Electron micrographs of sections through normal



Figure 1. The wts phenotype seen in mitotic recombination clones homozygous for the Df(3R)A177der20 deficiency, induced by gamma irradiation (1000 rads). Clones are marked by homozygous recessive Ki⁺, which produces normal (long, straight) bristles in a background of Ki^s heterozygous cells that produce short, curved bristles (Gausz et al. 1994). (a) Multiple clones on a wing, larva irradiated at 72 hr. (b) Three patches of mutant tissue (arrows) on a single leg, probably representing a single, split clone; larva irradiated at 48 hr. (c) A patch of mutant tissue forming a bulge on the tarsus; larva irradiated at 48 hr. (d) A clone on the surface of the leg, showing unusual rounded shape and polygonal cell outlines in the clone; larva irradiated at 84 hr.

integument show no discontinuity of the cuticle at cell boundaries (Fig. 3a), but within wts clones the cell boundaries are often marked by deep intrusions of cuticle (Fig. 3b). However, these intrusions do not separate cell membrane regions that are normally in contact; adherens junctions, normally found at the apical end of the lateral cell membrane (Poodry and Schneiderman 1970), are present at the bases of the cuticular intrusions, and septate junctions are present basal to the adherens junctions, as in normal cells (Fig. 3c). The presence of intact apical junctions indicates that the cellular defect in wts clones is not apical cell separation followed by cuticle deposition between the separated cells; rather, the apical ends of the cells are domed instead of flat as in normal epithelium. Deposition of cuticle over the domed apical surface produces the altered cuticular morphology.

Imaginal disc overgrowth in the wts^{P2} mutant

One of the P-element insertions in the wts gene, wts^{P2} , allows survival of homozygotes to the pharate adult



Figure 2. Shape and size of homozygous *wts* clones. Larvae simultaneously heterozygous for the mutation multiple wing hairs (*mwh*) and for the deficiency Df(3R)A177der20 were irradiated at 72 hr after egg laying. The outlines of mitotic recombination clones on the wing were traced with a camera lucida and measured with a compensating polar planimeter. (*a*) Control clones marked with *mwh*; mean area $3.6\pm0.4\times10^3 \ \mu\text{m}^2$; n=67. (*b*) wts clones; mean area $5.7\pm0.4\times10^3 \ \mu\text{m}^2$; n=154.



Figure 3. Ultrastructure of *wts* clones. (*a*) Electron micrograph of a section of an adult leg showing the smooth cuticle characteristic of the normal integument (c) overlying two epidermal cells. (*b*) Electron micrograph from the same section of the adult leg shown in *a*, in the region of a Df(3R)A177der20 clone. Cuticular intrusions (c) appear to invade between adjacent epidermal cells, but the presence of adherens junctions and septate junctions basal to the intrusions indicates that the cuticular intrusions are separating hypertrophied apical regions of the epithelial cells. (*c*) Higher magnification of the apical–lateral region of two cells within the Df(3R)A177der20 clone showing the intact septate (s) and adherens (a) junctions.

stage, and third-instar larvae of this genotype show remarkable hyperplasia of all of the imaginal discs, without noticeable effects on other tissues (Fig. 4). wts^{P2} homozygous imaginal discs (Fig. 5) show apical hypertrophy similar to that seen in wts^- mitotic recombination clones in adult tissue. Therefore, the defect leading to altered cuticle morphology is in the control of cell shape rather than in cuticle formation per se.

Deficiency mapping of wts

The position of the wts gene is defined genetically by

overlapping deficiencies on chromosome arm 3R (Fig. 6). The wts phenotype is produced after irradiation of larvae carrying the deficiencies Df(3R)A177der20, Df(3R)A177der25, Df(3R)A177der26, Df(3R)A177.X1, or Df(3R)P75A.A1. The phenotype is also produced by irradiating larvae heterozygous for Df(3R)A177der21 but only when such larvae also carry the duplication Dp(3;Y)A113 (Fig. 6). This result suggests that Df(3R)A177der21 deletes all or part of the wts gene, and that it also removes a more distal gene required for cell viability that is provided by the duplication *Dp*(3;Y)*A113*. The *Df*(3*R*)*A177der20* deficiency removes at least one additional gene, identified by the lethal P-element insertion P622, proximal to wts, whereas this is not the case for Df(3R)A177der21. Therefore, among these deficiencies Df(3R)A177der21 provides the closest breakpoint proximal to wts.

The translocation T(Y;3)A113, consisting of Dp(3;Y)-A113 and Df(3R)A113, is viable over Df(3R)A177der20, demonstrating that both wts and tailless (tll) are present on the translocation and are not disrupted or inactivated by it. Dp(3;Y)A113 rescues $tll^a/Df(3R)A177der20$ but does not rescue the wts phenotype produced by homozygous Df(3R)A177der20 in clones, indicating that its breakpoint is between wts and tll. In agreement with this conclusion, homozygous Df(3R)A113 fails to produce mitotic wts clones when combined with the telomeric duplication Dp(3;1)150P (Strecker et al. 1988) to cover the distal cell lethality, and Df(3R)A113 uncovers tll (Strecker et al. 1988).

The deficiency tll^e (Fig. 6) fails to produce the *wts* phenotype in mitotic recombination clones, which is consistent with both of its breakpoints being distal to the *wts* gene. Df(3R)P75A.A1 does produce the *wts* phenotype, and it complements both Df(3R)A113 and tll^e . Thus, the distal breakpoint of Df(3R)P75A.A1 could be either within or distal to the *wts* gene.

P-element mutations

To identify the wts transcription unit unambiguously by specific mutations, we carried out a P-element mutagenesis screen. The first screen was designed to detect all P-element insertions in the region of interest by the use of DNA probes and hence was independent of whether the insertions induced a phenotype. After extensive screening, one insertion, P509-19, was isolated that maps to the interval between the breakpoints of the deficiencies Df(3R)A113 and Df(3R)A177der21. This insertion does not produce a wts phenotype in mitotic recombination clones, and homozygous P509-19 flies are fully viable with no apparent phenotype. Therefore, P509-19 was used in a second screen for wts mutants by the induction of local hops or imprecise excisions. By this strategy, 45 mutants that generate a wts phenotype were obtained of which 32, designated $wts^{P1}-wts^{P32}$, retain some white⁺ activity of the P element, whereas 13, designated wts^{E1}-wts^{E13}, have lost white⁺ activity and are probably imprecise P-element excisions. All 45 lines are homozygous lethal and fail to complement either



Figure 4. Imaginal disc overgrowth in larvae homozygous for wts^{P2} . (a) Full-grown normal wing imaginal disc at 5 days after egg laying (AEL). (b) wts^{P2} homozygote at 5 days AEL. (c) wts^{P2} homozygote at 6 days AEL. (d) wts^{P2} homozygote at 8 days AEL. (e) Histological section of a disc comparable to the one in d, showing single-layered epithelial structure.

Df(3R)A177der20 or Df(3R)P75A.A1, and all of the alleles that have been tested fail to complement one another. All alleles except wts^{P2} produce embryonic or early larval lethality in homozygotes, suggesting that the wts gene also functions in embryogenesis.

Isolation and identification of the wts gene

The wts gene was isolated by a chromosomal walk extending in the proximal direction from a clone, S1.1, that mapped to 100B1,2 (Fig. 7). The proximal breakpoints of the deficiencies that delimit the region including the wts gene were mapped by in situ hybridization to polytene chromosomes and by whole-genome Southern analysis. In agreement with the complementation analysis (see Fig. 6), the region between the proximal breakpoints of Df(3R)A177.X1 and Df(3R)A113, which is expected to harbor the entire wts gene, also contains the proximal breakpoint of Df(3R)A177der21 at about its midpoint, thus identifying a ~25-kb region that includes at least part of the wts gene (Fig. 7). Neither breakpoint of Df(3R)P75A.A1 is present between the Df(3R)A177.X1and Df(3R)A113 breakpoints.

Reverse Northern analysis (data not shown) detected several transcribed genomic fragments in the relevant region of the chromosome walk. The most proximal of these corresponds to a gene encoding a protein with nine zinc fingers and a homeo domain, *zfh-1*, that had been shown previously to be included in the region immediately proximal to the Df(3R)A177der21 breakpoint (Fortini et al. 1991). This gene is distinct from *wts* based on analysis of the P-element insertion *P865*, a lethal insertion received from the *Drosophila* genome project. *P865* is located only 52 bp upstream of the 5' end of the longest *zfh-1* cDNA (Fig. 7) isolated previously (Fortini et al. 1991) and hence, is expected to owe its lethality to the inactivation of *zfh-1*. As this insertion complements Df(3R)A177der21 and fails to display the *wts* phenotype in mitotic recombination clones, we conclude that zfh-1 is not the wts gene.

The transcription unit immediately distal to zfh-1 is a more likely candidate for the wts gene. Therefore, sev-



Figure 5. Ultrastructure of the apical region of epithelial cells in normal (a) and wts^{P2} (b) imaginal wing discs. The apical microvillar surface (m) above the adherens junctions (a) is domed because of apical hypertrophy of the mutant cells, but relatively flat in normal cells.



Figure 6. Cytogenetic map of the 100A-100B chromosome region. Deficiencies and duplication: P75A.A1, y-ray-induced deficiency isolated in the FLP-FRT mitotic recombination screen; A177der- and A177.X1, deficiencies derived by γ -ray-induced loss of the P-element insertion P[lArB]A177; deficiency and duplication A113, reciprocal halves of the translocation T(Y;3)A113 (Lindsley et al. 1972); tll^e, tailless^e deficiency (Strecker et al. 1988). Arrows indicate that the duplication or deficiency extends beyond 100B. Cytology of the deficiencies was determined by examination of polytene chromosome squashes of deficiency over Ore-R chromosomes, except deficiency and duplication A113, which is from Strecker et al. (1988). Uncertainty of breakpoints is indicated by hatched boxes. Genes: P622, a P-element insertion causing recessive lethality (Berg and Spradling 1991); wts, the warts gene (P1-P32, presumed P-element insertion alleles of wts; E1-E13, presumed P-element excision alleles of wts); tll, the tailless gene (Strecker et al. 1988); EGX4, EGX8, and EGX13, lethals from J. Lengyel and J. Merriam (pers. comm.); dco, lethal(3)discs overgrown (Jursnich et al. 1990). All possible combinations of the various alleles and deficiencies shown were crossed to produce the complementation map. Arrows indicate that the deficiency may extend beyond the tested complementation groups. The warts phenotype is produced in mitotic recombination clones homozygous for Df(3R)P75A.A1, Df(3R)A177der20, Df(3R)A177der25, Df(3R)A177der26, Df(3R)A177der21 in combination with Dp(3;Y)A113, and by Df(3R)A177.X1.

eral cDNAs corresponding to this transcript were isolated from embryonic and imaginal disc libraries. Northern analysis showed the transcript identified by these cDNAs to be ~ 6 kb long and expressed throughout embryonic, larval, and pupal stages (data not shown). The gene is transcribed in the opposite direction to zfh-1, includes two large and five small introns, and extends over 17 kb of genomic DNA. It spans most of the region between the breakpoints of Df(3R)A177der21 and Df(3R)A113 (Fig. 7). Because of the formal possibility that the wts gene could lie within one of the two large introns in the 17-kb transcription unit defined by the cDNAs (Fig. 7), it was crucial to identify the wts gene by more direct methods.

The wts transcription unit was identified unambiguously by analysis of P-element insertions that give the wts phenotype in mitotic recombination clones. The flanking DNA sequences of several P-element insertions were determined and searched for overlaps with the 5.3 kb of combined cDNA sequences. This approach showed one P element, wts^{P14}, to be located in the untranslated trailer region, 120 bp upstream of the poly(A) addition site, and another, wts^{P2} , to be inserted 31 bp upstream of the longest cDNA (Figs. 7 and 8). The orientation of both P elements is opposite to that of the original P509-19 insertion. To extend the cDNA sequences maximally and determine whether the wts^{P2} insertion affects this transcript directly, a 5' RACE (rapid amplification of cDNA ends) experiment (Frohman 1990) was carried out with $poly(A)^+$ RNA from both third-instar larvae and embryos. Sequencing the 5' ends of the RACE products showed that the transcriptional start site is the same at both developmental stages and that wts^{P2} is located 38 bp within the 5' exon (Fig. 8). Whole-genome Southern analysis and sequencing of the DNA flanking the P elements obtained by plasmid rescue revealed that both mutant chromosomes, wts^{P2} and wts^{P14}, harbor a second P element in the 17-kb transcription unit. Two P elements on a given mutant chromosome might be the result of a local hop of the original P element from one chromatid onto its sister chromatid. The second P-element insertion on the wts^{P2} chromosome is identical with the original P509-19 insertion, which fails to produce a wts phenotype. Therefore, the insertion into the first exon is responsible for the wts^{P2} phenotype and clearly identifies the 17-kb transcription unit as the wts gene. The second P-element insertion in the wts^{P14} mutant is 73 bp upstream from, and has the same orientation as, the original P509-19 insertion. This insertion

might also contribute to the wts^{P14} phenotype. Most of the remaining $wts^{P1}-wts^{P32}$ mutations are deletions of DNA flanking the P509-19 insertion, usually in the proximal direction, without loss of distal P-element sequences. Several mutants, wts^{P3} , wts^{P23} , wts^{P25} , and probably wts^{P4} and wts^{P28} , correspond to perfect inversions of the original P509-19 insertion, indicating that the effect of the P-element insertion on expression of the wts gene depends critically on its orientation at this site.

The wts gene encodes a serine/threonine protein kinase

The entire wts cDNA sequence, from its 5' end defined by the RACE products to its 3' end at the poly(A) addiJustice et al.

Figure 7. Cloning and identification of the wts gene. (Top) Several deficiencies, P-element insertions, and a duplication are indicated with respect to a genomic EcoRI map (scale in kbp; positive direction oriented toward the telomere; broken lines indicate EcoRI sites of neighboring fragments whose order has not been determined), derived from a chromosomal walk between 100A1,2 and 100B1,2 (BL14.14 to S1.1 are inserts isolated from a Kr^{SB1}/CyO library in EMBL4). Shaded boxes delimit the regions that include the deficiency breakpoints. The proximal breakpoints of the three deficiencies shown at the top have not been determined but are located proximal (to the left) of the region covered by the arrows, whereas the distal breakpoint of Df(3R)A177der20 is located about 10 kb distal to the distal end of the walk shown. The positions of all P-element insertions have been mapped by DNA sequencing. Below the chromosomal walk, the extent and direction of transcription of zfh-1 (Fortini et al. 1991), wts, and tll (Pignoni et al. 1990) are indicated by arrows. The wts gene extends between the distal boundary of 100A1,2 and the proximal end of 100A5,6. (Bottom) A restriction map of the enlarged region including the entire wts gene, its intron/exon structure with the open reading frame (in black), and the location of the wts^{P2} and wts^{P14} insertions are shown. (B) BamHI; (R) EcoRI; (S) SalI; (X) XhoI.

tion site, is shown in Figure 8. It consists of 1136 nucleotides of untranslated leader sequence, an open reading frame encoding a protein of 1099 amino acids, and an untranslated trailer sequence of 927 nucleotides. Comparison of the cDNA with the corresponding genomic sequence reveals two large introns of ~5.5 kb each, which interrupt the cDNA in its 5' portion, and five small introns between 61 and 69 bp in length, all located in the carboxy-terminal coding region (Figs. 7 and 8). The open reading frame begins in the second exon (Fig. 8), and the homozygous viable P-element insertion *P509-19* is located in the middle of the first intron (Fig. 7).

Conceptual translation of the open reading frame (Fig. 8) predicts a protein of 121 kD. BLAST searches of the GenBank data base (Pearson and Lipman 1990) indicated a high level of similarity in the carboxy-terminal region to the catalytic domains of several serine/threonine protein kinases (see Discussion). The sequence also includes



an 87-nucleotide imperfect opa trinucleotide repeat (Wharton et al. 1985) encoding a glutamine-rich string. The amino-terminal region, outside the region of kinase homology, is rich in proline (12.7%) and serine (10.9%) but shows no significant sequence matches to current entries in GenBank.

Discussion

Loss or inactivation of both copies of the *wts* gene, encoding a putative serine/threonine protein kinase, leads to loss of control over the amount and direction of cell proliferation in developing imaginal discs, and also has dramatic effects on cell shape, leading to abnormal deposition of adult cuticle between epithelial cells. Because of its role in the control of cell proliferation and differentiation, we consider *wts* to be a tumor suppressor gene.

Figure 8. cDNA and protein sequence of the *wts* gene. The nucleotide and putative amino acid sequences derived from cDNA, 5' RACE, and genomic DNA sequences are shown. The protein kinase catalytic domain is shown by double underlining and the opa repeat by single underlining. The locations of the P-element insertions, wts^{P2} and wts^{P14} , are indicated after nucleotides 38 and 5240, respectively. The positions of the two large introns (5.5 and 5.6 kb) and of the five small introns (69, 63, 65, 61, and 62 bp) are marked by a vertical line below $\mathbf{\nabla}$, those of the 5' ends of the cDNA clones cBL9.1.3-3, cBL9.1.3-1, cBL9.1.3-2, cBL9.1.3-4, 4g, cBL9.1.3-5, and 3a (ordered according to decreasing cDNA length) by vertical arrows. All cDNAs extend to the poly(A) addition site shown, with the exception of 3a, which extends ~400 bp beyond it, and of cBL9.1.3-3, which ends after position 2102. The transcriptional start site is characterized by a 12-bp repeated sequence that includes an 8-bp palindrome (broken underline). Four polymorphisms attributable to the presence or absence of a few repeated base pairs (dotted underline) were found in the leader and trailer sequences of several genomic DNAs and cDNAs. The nucleotides and amino acids are numbered in the left and right margin, respectively.

w<u>ts</u>P2

120 480 600 GATAAATAATCCGACGACAAACAAAGCAGAAGCCTACACCGCGAGAAAGAGCAGTGCAAACACCAATTATCTTTATTGAGAGCAACAATATCAAGATCGAGATAATAAAGCATCCTAAAAC 720 840 960 21 1080 T_N 61 Λ Ν 1200 GAGAGO CACCTCTGAGATACACGGCGACCAACGGACGCAACGATGCACTTACTCCTGAC CAGAA 101 D D M D H H A K Q P M E P P P S A S P A P D V V I P P P A I ™CACCACGCCAAGCAGCCGATGGAGCCGCCACCCTCCCGCCTCCCGCCGCGGCGGGGGCGATACCGCCGCCGCCGCCA 14 Q P G A G S I S V S CAGCCCGGAGCCGGCTCCATATCCGTATCC 1320 141 JADTAADAAADAA 1440 GGTGTGGGCGTTGGAGTGGTGGGGTGTGGCGAACGGACGTGTGCCAAAGATGACGGCCCTAATGCCAAA 181 D G D S R T V G N P G G N G A 1560 s s D 22 G F v P Ρ R N 1680 ĸ♥ĸ R Ν R Р P Р p 0 R G Ν ٧ 0 0 261 Ν Т GAAGAACCCGCAGCAGCAGTTG 1800 TACGTGÃAGCGGCGATCACCGGCCCTGÃACÃACCGCCCGCCGCGGCGATAGCGCCACCCACTCAGCGAGGCÃ G G G 301 0 G Δ Α 1920 AGGCGGAGCCGGAGGAGCAGCACCGCCGCCGCCACCA 341 P S Y T A S M Q S R Q S P T Q S Q Q S D Y R K S P S S G I CCCAGTTACACGGCCTCCATGCAGTCGCGGCAGTCGCCCCACAAATCCCCAACAATCGGACTACAGGAAATCCCCGAGCAGTGGGAT 0 Q Q D R G G 2040 ATACTCGGCCACCTCGGCGGGCTCGCCGAGCCCC I T V S L P P A P L A K P Q P R V Y Q A R S Q Q P I I M Q S V K S T Q V Q K P V ATAACTGTGTCCCTGCCGCGCGCGCGCGCGCGGCGAAGCCACGAGCCACGAGCGCGAGGCGAGGCGGAGCGCGCGAGGGTGAAGAGCACGCCAGAGCCCCAAAGCCCGTG 381 2160 421 Ω 0 2280 461 Q Q A A A A A H Q Q Q H Q H Q Q S K P A T P T T P P L V G L N S K P N C L E P P CAACAGGCAGCAGCGGCGGCCCACCAGCAGCAGCACCAGCAATCCAAACCAGCAACGCCAACCACCACCGCCCTTGGTGGGTCTGAACAGCAAGCCAATTGCCTGGAGCCACCG 2400 0 0 0 0 ٧ 0 0 0 Q Q Q Q Q Q 501 0 0 0 0 0 0 0 0 0 v 0 0 2520 TCCTATGCCAAGAGCA AGCAGGTCCAGCAGCAGCAGGTGCAACAGCAGCAGCAACAGCAACAG R D Q Q K L A N G N P G R Q M L P P Acgggatcagcagaagctggccaacggaaatcctggccggcagatgcttccgccg 541 Ω D Q CAACTGCAGGCCI 2640 CCAGGCACAGGCTCAGAGGGAGCGGGATCAACGGGAGCGGG D 0 581 CCGCCTGCCAAA 2760 CCACCCATI 621 G 2880 661 R 0 0 Ε AGAGAACGTGATCAAGTCG 3000 н М Q 701 0 F к м к v G L Ρ D 0 0 CAAAAGGAGAGCAACTACATT 3120 TATCGCCAGCGCACGTATCGCAAGAATCAGCTGGAGAAGGAGATGCACAAAGTGGGACTGCCCGATCAGACCCAAATCGAGATGAGGAAAATGC<u>TGAA</u> к м KS G V G F G F v S к I D S 741 R D м F ٧ к 1 К Р I Α Α 3240 GCG TGGAGTGGGTGCATTTGG 781 A D V L K R N Q V A H V K A E R D I AGCGGACGTTCTCAAGCGGAATCAGGTGGCACACGTGAAGGCCGAGAGGGA<u>T</u>A D N 3360 ΤΤC N L Y F V M D Y I P G G D L M S L L I K L G TAATCTGTACTTTGTGATGGAGAGTACATACCAGGTGGTGATCTGATGTCGCTGCTCATCAAACTGGG 821 3480 861 v н К 3600 901 N GN H S R Q D S M E Y S E N G P K P TACTCCGAGAACGGACCCAAGCC 3720 CACCGTGCTGGAGAGG Y T Q L C D Y W S V GGTACACGCAGCTGTGCGACTACTGGAGCGTG L V G T P N Y I A P E V 941 Н G 3840 N S P L E T Q Q K V I N W E K T L H CCAACAGTCCGCTGGAAACGCCAACAAAAGGTCATCAACTGGG<u>AGAAAACGCTGC</u> 981 V G Q P P F L A I P P Q A E G V I L Y E M GGCGTCATCCTTTACGAGA 3960 TGACAAGCGGCTGGGCAAGAGCGTGGACGAGGTCAAGAGC F F K G I D F A D 1021 D Н D 4080 M R K Q K A P Y I P E I K H P T D T S N F D P V D P E K L R S N D S T M S S G D <u>ATGCGGAAGCAGAAAGCGCCCTACATACCGGAAATCAAGCACCCCAACGGACACCATCCAACTTTGATCCCGTGGATCCGGAGAAGCTGCGC</u>TCGAATGACTCCACCATGAGCAGCGGCGAT 1061 4200 1099 D V D Q N D R T F H G F F E F GATGTCGACCAGAATGACCGCACTTTCCACGGCTTTTTCGAATT 4320 GGATGCTCTCCATGTGCCCAACACCCAACACCCCGCCCCCGAATCATTGTTAGTCAAATAGTCACAAAAAGGGGATAGAAACCATTGAGTGGGCTTGCATTGTAAAGGAAGCGTGGCTATA 4440 4560 CATGAACTGAATAAAATATAAAACGGAGCCGAGTAGAGATGAAACGAGAGGAGGAGCGAGTCAGGACCTTCGACCTTTAACTGAACATAGTATATCCTTGTGCACTACTACTCTACCACAAATA 4680 TATATTTTTAAATTGTTAGAATTCAAAAGGGACCAACTGGAAATCGAACCTTTCTGGTGCTCAAAGCAAAGCAAAGCAAAACCAAAACGCCTTAAACTAAACGAGACGCGAATTTA 4800 CCCAACCACTTCACTCCTCTCTCTCCCACCCCGATCGGTGGCCGGATTCGAACTCGGCAGCTGGTTGCATCCGGCCACCCCATTGACTTCACAACTGAGATTGCGAGGTG 4920 5040 wtsP14

Figure 8. (See facing page for legend.)

A data base search with the conceptual translation of the wts gene sequence revealed a region very similar to the catalytic domains of several protein kinases (Fig. 9). These include the human myotonic dystrophy protein kinase (DM kinase; Mahadevan et al. 1993) and the Cot-1 protein kinase from N. crassa (Yarden et al. 1992). The amino acid sequence of the Wts protein kinase catalytic domain (using the boundaries used by Hanks et al. 1988) is 45.7% and 49.7% identical to the DM and Cot-1 kinase sequences, respectively. Wts also shows sequence similarity to some closely related predicted protein kinases from the plants Mesembryanthymum crystallinum (Fig. 9), Spinacia olaraca (B. Baur, K. Winter, K. Fischer, and K. Dietz, unpubl., GenBank accession nos. Z30329, Z30330), and Nicotiana tabacum (Y. Huang, J. Wan, K. Ko, and D.T. Dennis, unpubl., GenBank acces-

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sion no. X71057) and slightly less similarity to DBF20 (Fig. 9) (Toyn et al. 1991) and DBF2 (Johnston et al. 1990) cell cycle-regulated protein kinases of Saccharomyces cerevisiae. An expressed sequence tag from Caenorhabditis elegans predicts a partial protein with similarity to these protein kinases (Waterston et al. 1992). The next most closely related proteins are the cAMP-dependent protein kinases, represented in Figure 9 by the example that shows closest similarity (37.7% identity) to Wts, the $C-\alpha$ subunit of bovine cAMP-dependent protein kinase (Wiemann et al. 1992).

In general, kinases display alternating regions of high and low sequence conservation through the catalytic domain (Hanks et al. 1988). Eleven subdomains of high conservation have been described for the kinase catalytic domain (Hanks et al. 1988), and all of these are present in

> Figure 9. Sequence comparison of Wts and homologous proteins. The predicted amino acid sequences of Wts, human DM kinase (DM; Mahadevan et al. 1993), Cot-1 from N. crassa (Yarden et al. 1992), serine/ threonine protein kinase from M. crystallinum (M.c.; B. Baur, K. Winter, K. Fischer, and K. Dietz, unpubl.), yeast DBF20 (Toyn et al. 1991), and the C- α subunit of bovine cAMP-dependent protein kinase (PKA; Wiemann et al. 1992) were compared using the MACAW 2.0 program (Schuler et al. 1991). (a) The position of the putative catalytic domain (Hanks et al. 1988) is shown in black, and the percent amino acid identity with this region of the predicted Wts protein is shown in white. The regions outside the putative catalytic domain are crosshatched. The putative kinase domains of DM and PKA are shorter than those of the other proteins mainly because they lack the long insert between subdomains VII and VIII. (b) Multiple sequence alignment of the catalytic domains and the immediate flanking sequences (darkness of shading indicates significance of the match, and lowercase letters indicate no match with the other sequences). The 11 conserved subdomains (Hanks et al. 1988) are designated by Roman numerals. Residues that are almost invariant among protein kinases (Hanks et al. 1988) are indicated by asterisks (*), and those where residues of similar structure are found in almost all protein kinases are indicated by dots below the alignment (\bullet) . Residues that characterize the Wts subfamily and distinguish it from its closest relatives, the cyclic nucleotide-dependent protein kinase subfamily (Hanks et al. 1988) represented here by PKA, are indicated by an arrow (solid, invariant; open, almost invariant) above the alignment.

Wts and its closest homologs (Fig. 9). Furthermore, all of the amino acids that are perfectly conserved in functional serine/threonine kinases are present in the proteins of this subfamily (Fig. 9). These residues are involved in ATP binding, proton transfer, Mg^{2+} binding, and substrate specificity. The nucleotide-binding consensus sequence Gly-X-Gly-X-X-Gly/Ser is present in subdomain I of Wts and the other related kinases (Fig. 9). The serine/threonine kinase-specific consensus sequences (Hanks et al. 1988) are well conserved in subdomains VI and VIII.

The predicted Wts protein and its close relatives form a new subfamily of kinases that are more closely related to each other in sequence than they are to other protein kinases. The 15 residues that distinguish them from their closest relatives, the cyclic nucleotide-dependent kinases, are indicated in Figure 9. The human DM kinase (Dunne et al. 1994) and the yeast DBF2/DBF20 kinases (Toyn and Johnston 1994) have been shown directly to have serine/threonine protein kinase activity, but their cyclic nucleotide dependence has not been reported. The similarities among the Wts-related kinases and the high degree of conservation of features critical to kinase function imply that these proteins may act in common pathways in vertebrates, invertebrates, and plants.

The similarity between the Wts and Cot-1 proteins is intriguing because the Neurospora and Drosophila mutations both cause local cellular hypertrophy, indicating that their normal functions are required to maintain cell shape. The cot-1 mutant phenotype is characterized by local swellings of the hyphae that later grow into multiple ectopic hyphal branches (Yarden et al. 1992) while the wts phenotype is characterized by apical hypertrophy of imaginal disc cells. In Drosophila, where the affected cell population is growing by proliferation, the gene product is also required for control of the amount of cell proliferation and the direction of clone growth, which is presumably a reflection of oriented cell divisions in the growing imaginal disc. It is conceivable that the latter effects are indirect results of the effect on cell shape, which might interfere with the signaling events that control cell proliferation. Alternatively, Wts may be involved directly in a signal transduction mechanism that controls both cell shape and cell proliferation. In either case, identification of the substrates of this protein will provide a new way of investigating the control of cell shape and proliferation. Recent work (Salvatori et al. 1994) indicates that the DM kinase is a peripheral membrane protein, suggesting that its substrates may include membrane or membrane-cytoskeletal proteins.

The most interesting homology of Wts is with its human homolog DM kinase. Amplification of trinucleotide repeats in the 3' trailer region of the DM kinase gene is responsible for myotonic dystrophy, the most common form of adult-onset muscular dystrophy in humans (Brook et al. 1992; Fu et al. 1993; Mahadevan et al. 1993). The severity is correlated with the degree of amplification of the trinucleotide repeats (Tsilfidis et al. 1992), which typically increase in number in successive generations (Mahadevan et al. 1992; Redman et al. 1993) and cause loss of DM kinase mRNA (Carango et al. 1993). Although the open reading frame of the *wts* gene includes an opa trinucleotide repeat (Wharton et al. 1985) encoding a stretch rich in glutamine, there is no evidence that these domains undergo amplification or are responsible for mutant phenotypes of the genes in which they are found.

The muscular dystrophy phenotype appears unrelated to the abnormalities we have described in mutant Drosophila. However, myotonic dystrophy is a dominant disorder seen in heterozygotes, whereas the Drosophila mutations are recessive and produce either the clonal tumor phenotype or the disc overgrowth phenotype only when both normal copies of the gene are lost. The phenotype caused by homozygous loss of function of DM kinase has not been reported, but an intriguing connection is suggested by the many reports of epithelial and other tumors in myotonic dystrophy patients. At least 18 myotonic dystrophy patients have been described with pilomatrixomas, tumors arising from hair follicle cells (Cantwell and Reed 1965; Kopeloff et al. 1992). Multiple pilomatrixomas, very rare in the general population, have been described in dystrophic individuals and in their otherwise asymptomatic relatives (Harper 1971; Chiaramonti and Gilgor 1978; Street and Rogers 1991; Kopeloff et al. 1992). Myotonic dystrophy patients have also been described with neuroendocrine tumors of neural crest origin (Reimund et al. 1992) including neurofibromatosis (Kissel and Arnould 1954; Ichikawa et al. 1981; Rosenberg et al. 1988), multiple endocrine adenomatosis type 2A (Rosenberg et al. 1988), parathyroid adenomas (Harada et al. 1987), and multiple carcinoid tumors of the small bowel (Reimund et al. 1992). Our results raise the possibility that somatic loss of heterozygosity of the DM kinase gene might contribute to the development of these tumors. Moreover, because of the high conservation of gene networks during evolution (Noll 1993), a study of the genes that interact with wts might shed light on the molecular mechanisms leading to myotonic dystrophy.

Materials and methods

General procedures

Standard procedures such as the isolation of genomic DNA, the construction and screening of genomic libraries, chromosomal walking, whole-genome Southern analysis, in situ hybridization to salivary gland chromosomes, isolation, and Northern analysis of $poly(A)^+$ RNA, and reverse Northern analysis were carried out essentially as described (Frei et al. 1985; Kilchherr et al. 1986). The DNA sequences flanking P-element insertions were recovered by plasmid rescue (Pirrotta 1986).

Generation of deficiencies

Most of the chromosome deficiencies defining the wts gene (Df(3R)A177der20, Df(3R)A177der21, Df(3R)A177der25, Df(3R)A177der26, and Df(3R)A177.X1; see Figs. 6 and 7) were recovered as γ -ray-induced losses of the ry^+ P-element insertion, P[IArB]A177, kindly provided by Hugo Bellen (Baylor College of Medicine, Houston, TX). Homozygous P[IArB]A177 flies were irradiated with 4000 rads of gamma rays, and ry^- F₁ progeny were selected. After establishing a balanced stock, each P[lArB]A177-derived chromosome was tested for effects on mitotic clone growth by inducing mitotic recombination in heterozygous larvae at a series of stages. Each of these deficiency chromosomes was also tested for complementation with other mutations, deficiencies and duplications in the 100A-100B interval (Fig. 6). In some cases, deficiencies were tested for their effects on mitotic clone growth in the presence of the duplication Dp(3; Y)A113. We also recovered an informative deficiency using a direct screening procedure to identify mutations of tumor-suppressor genes based on their phenotype in mitotic recombination clones in the F_1 generation. This procedure takes advantage of transgenic flies in which a yeast site-specific FLP recombinase gene has been placed under the control of a Drosophila heat shock promoter and integrated into the Drosophila genome (Golic and Lindquist 1989). FLP recombinase target sequences (FRTs), also derived from yeast and required in homozygous form as targets for the FLP recombinase, have been integrated into several proximal sites on Drosophila chromosomes (Golic and Lindquist 1989). Heat shock of flies carrying the FLP recombinase construct and homozygous for an FRT target site leads to mitotic recombination clone frequencies of well over one per fly (Golic 1991). Males homozygous for an FRT on the proximal part of chromosome arm 3R were mutagenized with 4000 rads of y rays and crossed to females carrying the same FRT insert on 3R, as well as the FLP recombinase gene on the X chromosome. The resulting larvae were heat-shocked for 1 hr at 37°C at 3.5 days (third larval instar) to induce mitotic recombination in the developing imaginal discs. Adult progeny were selected that had any outgrowths, and these were tested by crossing to Df(3R)A177der20, a deficiency known to include the wts gene. Those chromosomes that failed to complement Df(3R)A177der20 were then retested for the wts phenotype in γ radiation-induced mitotic clones and in FLP-FRT recombination clones. From this screen we obtained the deficiency Df(3R)P75A.A1.

P-element mutagenesis

In a large-scale phenotype-independent screen for P-element insertions (N. Walter, M. Jenni, A. Fritz, O. Zilian, M. Noll, and E. Hafen, unpubl.) into the cloned chromosomal region delimited by the proximal breakpoints of Df(3R)A177.X1 and Df(3R)A113, a single P-element insertion, P509-19, was obtained (Fig. 7), which was homozygous viable and without apparent phenotype. This element, a P-lacW (Bier et al. 1989), was remobilized in a subsequent screen for insertions in the wts gene by taking advantage of the fact that P elements preferentially insert into nearby sites ("local hops"; Tower et al. 1993; Zhang and Spradling 1993). This was achieved by crossing w¹¹¹⁸;P509-19/P509-19 flies to w flies carrying the stable genomic transposase source $\Delta 2$ -3, located at 99B on the balancer chromosome TMS (Robertson et al. 1988). The w; P509-19/ TMS, Sb $\Delta 2$ -3 female offspring were mated with w males, and their larval progeny were irradiated at 3-4 days after egg laying with 1000 rads of γ rays to induce mitotic recombination of the potentially wts⁻ third chromosomes. The resulting adults were then examined for the presence of epithelial tumors similar to those produced by the wts deficiencies. The frequency of tumorous flies among flies of the appropriate genotype was 0.72% ($n \sim 20,000$). Then we tested the third chromosomes from these tumorous flies for complementation with Df(3R)A177der20 or Df(3R)P75A.A1 and recovered the mutant third chromosomes in balanced stocks. All of the tested chromosomes fail to complement either Df(3R)A177der20 or Df(3R)P75A.A1 (Fig. 6) and all of them show the warts phenotype in mitotic recombination clones; therefore, we designate them wts^- . Thirty two of these wts^- chromosomes (designated $wts^{P1}-wts^{P32}$) show retention of some white⁺ activity of the P element, producing variegated eyes or eye colors ranging from yellow to red. These could represent insertions of the P element into the wts gene, presumably at different sites in view of the differences in white⁺ expression. Alternatively, they could represent mobilization to other sites on the third chromosome accompanied by imprecise excision of P509-19. Thirteen $wts^$ chromosomes (designated $wts^{E1}-wts^{E13}$) have lost white⁺ activity and are presumed to represent imprecise excisions of the P element or insertions associated with complete inactivation of white⁺ in the eye.

Isolation of cDNA clones and RACE

Preparation of cDNA libraries from $poly(A)^+$ RNA from adult *Oregon-R* females (Watson et al. 1992), from 4- to 8-hr-old embryos (Schneitz et al. 1993), and from imaginal discs was as described, and isolation of cDNA clones 3a (from adult females), 4g (from imaginal discs), and cBL9.1.3-1, cBL9.1.3-2, cBL9.1.3-3, cBL9.1.3-4, cBL9.1.3-5 (from embryos) was carried out according to standard procedures. To clone a cDNA that includes the transcriptional start site of the *wts* mRNA, the 5' RACE technique was applied to poly(A)⁺ RNA from 8- to 12-hr-old embryos or from late third instar larvae, using the 5' Amplifinder RACE kit and following the instructions of Clontech.

DNA sequencing

DNA sequencing by the dideoxynucleotide method was carried out by PCR sequencing (Life Technologies) using both external (T7 and T3) and internal primers, synthesized by Operon Technologies, or with a DNA sequencer model 373A using dye terminators (Applied Biosystems Inc.).

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Note added in proof

The sequence data presented in this paper have been deposited into the GenBank/EMBL data libraries under accession number L39837.

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