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The homeobox-containing gene *Wariai* regulates anterior-posterior patterning and cell-type homeostasis in *Dictyostelium*

Zhihua Han and Richard A. Firtel*

Department of Biology, Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0634, USA
*Author for correspondence (e-mail: rafirtel@ucsd.edu)

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SUMMARY

We have identified a *Dictyostelium* gene, *Wariai (Wri)*, that encodes a protein with a homeobox and seven ankyrin repeats; both domains are required for function. A null mutation results in a more than doubling of the size of the prestalk O (pstO) compartment, one of the anterior prestalk compartments lying along the anterior-posterior axis of the migrating slug. There is a concomitant decrease in the more posterior prespore domain and no change in the more anterior prestalk A (pstA) and prestalk AB (pstAB) domains. *wri* null cells also have a morphological defect consistent with an increase in the pstO cell population. *Wri* itself is preferentially expressed in the pstA but not the pstO compartment, raising the possibility that *Wri* regulation of pstO compartment size is non-autonomous. Analysis of chimeric organisms is consistent with this model. Development in *Dictyostelium* is highly regulative, with cells within the prestalk and prespore populations being able to transdifferentiate into other cells to maintain proper cell-type proportioning. Our results suggest that *Wri* controls cell-type proportioning, possibly by functioning as a negative regulator of a pathway mediating pstO cell differentiation and controlling the mechanism of homeostasis regulating the size of one or more of the cell-type compartments. Our results also suggest that homeobox gene regulation of anterior-posterior axis patterning may have evolved prior to the evolution of metazoans.

Key words: *Dictyostelium discoideum*, Homeobox, Ankyrin repeat, *Wariai*, Anterior-posterior patterning

INTRODUCTION

In *Dictyostelium*, the multicellular organism is formed by the chemotactic aggregation of up to 10^5 individual cells (Firtel, 1995; Van Haastert, 1995; Chen et al., 1996). This is mediated by cAMP that interacts with cell surface, G-protein-coupled receptors, activating a series of downstream signaling pathways that control directed cell movement, relay of the cAMP signal and gene expression. Upon mound formation, rising levels of extracellular cAMP trigger a transcriptional cascade regulated through the transcription factor GBF and a cAMP receptor-mediated signaling pathway to initiate morphogenesis and cell-type differentiation (Schnitzler et al., 1994, 1995; Firtel, 1995).

In wild-type organisms, cell-type differentiation and morphogenesis are tightly coupled. Shortly after mound formation, a tip emerges and the organism elongates to form a cylindrical first finger that falls over onto the substratum to form a migrating slug or pseudoplasmodium (Loomis, 1975). Under appropriate conditions, culmination initiates and the organism differentiates into a mature fruiting body containing a spore mass on top of an elongated vacuolated stalk sitting on a basal disc. Spatial patterning within the migrating slug is well-understood. For the most part, spatial patterning in the slug is organized along an anterior-posterior axis. The anterior ~20% of the slug is composed of three distinct classes of prestalk cells (Williams and Morrison, 1994; Early et al., 1993; Jermyn et al., 1989). The very anterior contains an internal core of prestalk AB (pstAB) cells. This lies in an anterior domain constituting ~10% of the organism composed of prestalk A (pstA) cells followed posteriorly by a domain of ~10% of the slug composed of prestalk O (pstO) cells. The posterior ~80% of the organism represents the prespore domain (Takeuchi and Sato, 1965; Krefft et al., 1984; Gomer et al., 1986; Haberstroh and Firtel, 1990). Within this domain and also scattered throughout the entire organism is another population designated anterior-like cells (ALCs) that have many properties of prestalk cells (see below; Devine and Loomis, 1985; Sternfeld and David, 1982). The very posterior of the slug (rearguard zone) contains an enrichment of ALCs that are lost as the slug migrates (Esch and Firtel, 1991; Sternfeld, 1992). Recent analysis by the Williams laboratory suggests that the prestalk A and pstO cells arise independently as the mound is formed and differentially sort during the process of tip formation (Early et al., 1995). The induction of pstA and pstO cells and subsequent induction of the pstAB cells requires the morphogen DIF in addition to a prior cAMP signal with pstA and pstO cells having a differential sensitivity to DIF (Early et

al., 1995; Morris et al., 1987; Town et al., 1976; Williams et al., 1987; Jermyn et al., 1987; Berks and Kay, 1990). The expression of prestalk-specific genes is mediated by a STAT transcription factor (Kawata et al., 1997); however, the role of DIF in potentially regulating STAT function is not known. Prespore cells arise independently within the mass of the mound (Haberstroh and Firtel, 1990; Krefft et al., 1984; Williams et al., 1989). Differential cell sorting of the various prestalk cell populations gives rise to the anterior-posterior patterning observed within the slug (Esch and Firtel, 1991; Traynor et al., 1992; Siegert and Weijer, 1995).

Cell-type patterning is independent of the size of the organism, which can range over >3 orders of magnitude from <100 cells to ~10^5 cells (Loomis, 1975; Raper, 1940; Bonner and Slifkin, 1949; Bonner, 1944). In contrast to that in metazoans, cell-type differentiation in *Dictyostelium* is plastic up to the time of culmination. Classic experiments by Raper showed that if slugs are cut, both the anterior and posterior regions, which preferentially contain prestalk and prespore cells, respectively, give rise to complete organisms with normal spatial patterning if slugs are given time to migrate (Raper, 1940). This has been generally called transdifferentiation. Additional studies in which individual cell types were 'tagged' using reporter constructs have shown that, even in wild-type slugs, there is transdifferentiation of prespore to anterior-like cells, and anterior-like cells (ALCs) are in equilibrium with the pstO population, which in turn is in equilibrium with the pstA population [psp<=>ALC<=>pstO<=>pstA] (Abe et al., 1994). These observations, taken in combination with the knowledge that spatial patterning is independent of the size of the organism, suggest the existence of highly regulated homeostatic mechanisms that control both the initial induction and maintenance of cell-type proportioning. Both of these processes are thought to be regulated in part by cAMP emitted from an oscillator in the anterior part of the organism combined with molecular mechanisms to differentially regulate the level of DIF in different parts of the organism (Firtel, 1995; Siegert and Weijer, 1995; Brookman et al., 1987; Kay, 1992). While some mutants have been identified that affect the proportions of different prestalk cell-types or the ratio of prestalk to prespore cells (MacWilliams et al., 1985; MacWilliams and David, 1984; Wood et al., 1996), little is understood about the actual molecular mechanisms controlling these processes.

Homeobox-containing (Hbx) transcription factors have been identified in most if not all phyla of eukaryotes (McGinnis et al., 1984; Scott and Weiner, 1984; Burglin, 1995). In metazoans, Hbx-containing proteins are known to be involved in regulating the anterior-posterior body plan and in the specification of certain cell types and anterior-posterior compartments (McGinnis and Krumlauf, 1992). In organisms from fly to man, many of these genes control the formation of the anterior-posterior axis. We have identified two homeobox-containing genes in *Dictyostelium*, *DdHbx-1* (named *Wariai*) and *DdHbx-2*, both of which are developmentally regulated and preferentially expressed starting at the mound stage in response to cAMP and require the transcription factor GBF. In addition to the Hbx domain, the encoded *Wariai* ORF contains seven ankyrin repeats (similar to IκB) in the C-terminal half of the protein. Our analysis suggests that *Wariai* is involved in regulating the size of the pstO compartment. *wariai* null cells show at least a two-fold increase in the pstO compartment by regulating the posterior boundary of this compartment. There is a concomitant decrease in the prespore domain and no observable change in the pstA or pstAB domains. *wariai* null cells also have a morphological defect consistent with an increase in the prestalk population. We show that *Wariai* is preferentially expressed in the pstA cells and ALCs with little, if any, expression in pstO cells. Our results suggest that *Wariai* may be a negative regulator of pstO differentiation and may control the equilibrium between pstO, anterior-like cells (ALCs) and pstA cells, and thus regulate the homeostatic mechanism controlling spatial proportioning. Both the homeobox and ankyrin repeats of *Wariai* are necessary for *Wariai* function. *ddhbx-2* null cells have no overt phenotype but the gene appears to accentuate the *wariai* null phenotype in cells in which both genes have been disrupted. These results suggest that, in *Dictyostelium*, homeobox genes may also control cell-type differentiation and spatial proportioning along the anterior-posterior axis, possibly by regulating equilibrium between various prestalk populations and prespore cells. *Wri* may represent the earliest evolutionary example of a homeobox-containing gene regulating anterior-posterior axis patterning, suggesting that this mechanism to control spatial patterning in multicellular organisms may have evolved prior to the evolution of metazoans.

MATERIALS AND METHODS

PCR and cDNA cloning

We utilized a single degenerate primer PCR method to clone potential homeobox-containing genes. Two degenerate oligonucleotides were designed by reference to conserved region of amino acids 48-54 in the homeodomain as described in the text and used with the T3 sequencing primer to amplify potential homeobox-containing sequences from a λZap cDNA library made from RNA isolated from cells between 8 and 16 hours of development (Schnitzler et al., 1994). Oligonucleotide 1, 5′-C(A,G)(T,A)C(T,G)(A,G)TT(T,C)TG(A,G)A-ACCA; oligonucleotide 2, 5′-(T,A)C(T,G)(T,A)C(T,G)(T,A)T-T(A,T)(C,G)(T,A)(A,G)AACCA. *DdHbx-1* (*Wri*) was identified in the PCR products from oligonucleotide 1, while *DdHbx-2* was identified in the PCR products from oligonucleotide 2. The T3 primer, which hybridizes to the N-terminal end of the polylinker region of the library, initiates from the 5′ end of all cDNA clones. *Taq* DNA polymerase from Gibco BRL was used. The reactions were performed with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl, 4 mM dATP, 3 mM dCTP, 3 mM dGTP, 4 mM dTTP, 0.5 μg of each primer, and 0.1 μl of cDNA library with a titer of ~10^{10}/ml. The PCR reaction steps included denaturation at 94°C for 2 minutes; then 40 rounds of denaturation at 94°C for 0.5 minutes, annealing at 37°C for 1 minute, and extension at 72°C for 1.5 minutes. The final extension step was at 72°C for 7 minutes. The PCR fragments generated were subcloned into Bluescript SK and sequenced. Those fragments that encode potential homeobox were used as probes for screening full-length cDNA from the same λZap library. Approximately 10^6 plaques were screened.

Cell culturing and molecular biology techniques

Cell culturing, transformation of *Dictyostelium* cells and subsequent analysis, and RNA and Southern blot analyses are all standard and were performed as previously described (Mann and Firtel, 1987; Howard et al., 1988; Datta and Firtel, 1988; Dynes et al., 1994). For all experiments, clonal isolates were obtained and more than one clone was examined.

Plasmid constructs

For the *Wri* knockout construct, the *Wri* cDNA clone was digested with *Nde*I to linearize the DNA at the *Nde*I site located within the homeobox domain. The DNA was blunt-ended with Klenow fragment and a *Bgl*II linker was ligated into the site. Either the 1.3 kb *Bam*HI fragment containing the blasticidin-resistant (*Bsr*) gene cassette (Sutoh, 1993) or the 3.5 kb *Bam*HI fragment containing the auxotrophic marker *Thy1* cassette (Dynes and Firtel, 1989) was cloned into this site. The *Bsr* construct was digested with *Pst*I and *Hin*dIII, then electroporated into wild-type KAx-3 cells. The *Thy1* construct was digested with *Sma*I and *Stu*I, then electroporated into JH10 cells (*thy1* null cells) (Mann and Firtel, 1991). Homologous recombination events were identified by genomic Southern blot analysis on randomly selected clones.

For the *DdHbx-2* knockout construct, a *Bam*HI site was created inside the homeobox by PCR. The original cDNA was subcloned into the *Eco*RI and *Xba*I sites of Bluescript SK, placing the N-terminal of the cDNA near the T7 primer. Two PCR reactions were performed to create an internal deletion and a *Bam*HI site. One used the T7 sequencing primer and a *DdHbx-2*-specific primer: 5′-GTTTGGATCCAATAAGACATACCTAAACG. The product was digested with *Eco*RI and *Bam*HI. The other fragment was amplified using the T3 sequencing primer and the *DdHbx-2*-specific primer: 5′-GTTTTGGATCCGTTCTCAAATAAACGTCAAG-OH. This product was digested with *Bam*HI and *Xba*I. The two fragments were simultaneously ligated into the *EcoRI* and *Xba*I sites of Bluescript. This procedure deleted amino acids 43 to 48 in the homeodomain and created a *Bam*HI site in their place. Either the *Bsr* gene cassette or the *Thy1* gene cassette was inserted into the new *Bam*HI site. The Bsr construct was digested with *Eco*RI and *Xba*I and electroporated into wild-type KAx-3 cells. The *Thy1* construct was digested with *Eco*RI and *Xba*I and electroporated into JH10 cells. Homologous recombination events were identified by genomic Southern blot analysis. See Fig. 1.

The *WriΔAnk* construct carrying an in-frame deletion in the ankyrin repeats was created by digestion of the cDNA with *Sty*I followed by religation of the DNA. The construct has a deletion of amino acids 403 to 628, which includes 6 of the 7 ankyrin repeats. See Fig. 1.

The *WriΔHbx* construct carrying an in-frame deletion of amino acids 12 to 38 in the homeobox was created using PCR. Specifically, the *Eco*RI fragment of the *Wri* cDNA was subcloned into Bluescript, which served as the PCR template. The two primers used for this PCR were 5′-GTTTACTAGTATTATTTTCTAAAAGATGGCATCA-3′ and 5′-GTTTCATATGGTCTGGTGATGTTCTCTTTCT-3′. The PCR product was digested with *Spe*I and *Nde*I, then subcloned back into *Wri* cDNA to create *pWriΔHbx*. This procedure also deleted 220 bp of 5′ untranslated sequence immediately upstream of the ATG initiation codon.

To express the two deletions constructs and the complete *Wariai*, the ORF-containing inserts were cloned into the *Spe*I and *Xho*I sites of a *Dictyostelium* expression vector downstream from the Act15 promoter (Dynes et al., 1994). *DdHbx-2* expression vectors were made using similar constructs.

Accession number for *Wariai* is AF036171 and AF036170 for *DbHbx-2*.

Cloning and analysis of the *Wariai* promoter

In *Dictyostelium*, promoters reside within 1 kb of the coding region. To clone the promoter, a pUC vector was inserted into the *Wri* gene by homologous recombination and then excised with 2.5 kb of upstream sequences. Specifically, *pUCBsrΔBam*HI, which consists of the 1.3 kb *Bsr* cassette and *pUC118*, was introduced into *Wri* in the genome by homologous recombination. To do this, *pUCBsrΔBam*HI was inserted into the *Nde*I and *Eco*RI sites of *WriΔHbx*. This created a deletion of amino acids 173 to 244 in the *Wariai* ORF, including amino acids 12 to 60 of the 60 amino acids homeodomain. The deleted sequences were replaced by the Bsr/pUC118. The resulting DNA was digested with *Spe*I and *Hin*dIII, then electroporated into wild-type KAx3 cells. Homologous recombination events were identified as outlined above. Then genomic DNA were purified, digested with *Hinc*II, self-ligated and transformed into supercompetent *Epicurian E. coli* cells (Stratagene). The rescued plasmid contains 2.5 kb upstream of the *Wri* coding region. See Fig. 1 for maps.

To make the *Wri* promoter-*lacZ* construct, PCR was used to create an in-frame fusion between the promoter and the *lacZ* gene. The *Hin*dIII-*Xho*I fragment from the rescued plasmid was subcloned into Bluescript SK *Hin*dIII and *Xho*I sites and served as a template for PCR. The primers used in this PCR were the T3 sequencing primer and 5′-GTTTAGATCTCATAACAATTGATGCCATCTT. The PCR product was digested with *Hin*dIII and *Bgl*II, and ligated into pDdGal-17 combined with the remainder of the promoter region (the *Hin*cII to *Hin*dIII part of the promoter region). This process created an in-frame fusion in which the complete 2.5 kb promoter region plus sequences coding for the first six amino acids joined to the multiple linker region are in-frame with the *lacZ* coding sequences.

Histochemical and neutral red staining

Histochemical staining experiments for β-gal and β-gluc activity were performed as previously described (Esch and Firtel, 1991; Jermyn and Williams, 1991; Mann et al., 1994). Cells were plated for development on 25 mm Millipore filters (Type HA, 45 μm from Millipore). At a specific developmental stage, the organisms were fixed briefly in Z buffer with 0.5% glutaraldehyde and 0.05% Triton X-100, then stained in Z buffer containing 2.5 mM $K_3Fe(CN)_6$, 2.5 mM $K_4Fe(CN)_6$ and 1 mM X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). For β-galactosidase and β-glucuronidase (β-*Gus*) double staining, X-gal was replaced by 2 mM X-GlcA (5-bromo-4-chloro-3-indolyl-β-D-Glucuronide, Sigma) and 1 mM salmon-β-D-gal (magenta-gal) (Early et al., 1993; Mann et al., 1994). X-GlcA gives blue color and salmon-β-D-gal gives red color.

For neutral red staining, log phase cells were washed several times with 12 mM Na/K buffer (pH 6.2) and incubated with 4 μg/ml neutral red in 40 mM phosphate buffer (pH 7.2), 10 minutes on ice followed by 5 minutes at room temperature. Then cells were washed three times in 12 mM Na/K buffer (pH 6.2) and plated on Na/K agar plates for development. Pictures were taken of migrating slugs.

RESULTS

In order to identify potential homeobox-containing genes, we implemented a ‘single primer’ PCR approach in which we used one degenerate primer from the highly conserved region near the C terminus of the 60 amino acid homeobox domain [amino acids 48-54; WFQNRR(M/A); WFSNRR(R/S)] (Burglin, 1995) combined with a PCR primer complementary to the polylinker used in construction of a λZapII library to identify *Dictyostelium* homeobox-containing genes. Two Hbx-directed degenerate oligonucleotides were designed, taking into consideration the sequence of various subclasses of metazoan homeoboxes as well as those identified in plants and yeast (Burglin, 1995; see Materials and Methods). PCR amplification of a λZapII library made from RNA isolated from cells between 8 and 16 hours in development (Schnitzler et al., 1994) identified two potential homeobox-containing clones in the initial sequencing screen of 25 randomly selected clones. These cloned PCR products were then used to screen the λZap library for full-length cDNA clones. The derived amino acid sequence of both of these ORFs is presented in Fig. 1. Visual sequence comparison of the domain identified in the PCR clones and BLAST (Altschul et al., 1990) searches identified a homeobox domain in both *DdHbx-1* and *DdHbx-2*. Because *DdHbx-1* regulates cell-type proportioning

(see below), we have named this gene *Wariai* (*Wri*), the Japanese word for proportioning or ratio. Comparison to a consensus homeobox (Hbx) sequence (Burglin, 1995) is shown in Fig. 1A. In addition, *Wariai* also contains seven ankyrin repeats in the carboxy-terminus that are most closely homologous to those found in mouse ankyrin (Birkenmeier et al., 1993; Fig. 1A). Like many other *Dictyostelium* proteins (Burki et al., 1991; Mann and Firtel, 1991; Pitt et al., 1992), *Wariai* and DdHbx-2 contain runs of repeated amino acids, including a long, very asparagine-rich region near the N terminus of *Wariai* and a more unusual glycine-rich-containing region followed by a region containing alternating histidine and asparagine residues. The DdHbx-2 sequence shows predominantly long homopolymer regions and stretches of hydrophobic amino acids in addition to the homeobox domain. The *Wri* homeobox shows strongest homology to the Hbx of the tapeworm gene *EgHbx4* (accession no. JC1389). Neither of the two *Dictyostelium* genes are members of any known homeobox gene family (Burglin, 1995).

Developmental RNA blot hybridization (Fig. 2) shows both genes are developmentally regulated: expression of *DdHbx-2* is first detected and that of *Wariai* rises significantly at 8 hours, the time of mound formation, when there is a developmental switch from aggregation to multicellular differentiation and just prior to cell-type differentiation (Firtel, 1995). No expression of *DdHbx-2* or increased expression of *Wri* was observed in *gbf* null cells in which the transcription factor GBF, which is essential for differentiation past the mound stage, is knocked out (Schnitzler et al., 1994), indicating *Wri* and *DdHbx-2* are dependent on GBF function for a high level of expression during the multicellular stages. Both genes are induced in suspension in response to cAMP (data not shown), as would be expected for genes preferentially induced at the onset of mound formation.

The morphological phenotypes of *wri, ddhbx-2* and *wri/ddhbx-2* null strains

Wariai was disrupted by homologous recombination in two independent strains: (1) wild-type KAx-3 cells using the Bsr (blasticidin) dominant drug selectable marker, and (2) in the *thy1* null strain JH10, which exhibits a wild-type pattern of development, using the Thy1 (thymidine) auxotrophic marker (Dynes and Firtel, 1989; see Materials and Methods). Multiple disrupted strains made using both selections were identified by Southern blot analysis (data not shown) of randomly selected, independent clones. All showed the same phenotype and no expression of *Wri* transcripts (data not shown). *wri* null cells aggregated and formed mounds normally when plated for development on non-nutrient agar. Approximately 30% of the organisms arrest at this stage, while the remainder proceed through development and form fruiting bodies with an enlarged ‘basal disc’ and lower portion of the stalk, suggesting there is an increase in the number of basal disc and stalk cells (Fig. 3D,F), compared to wild-type cells (Fig. 3B). In addition,

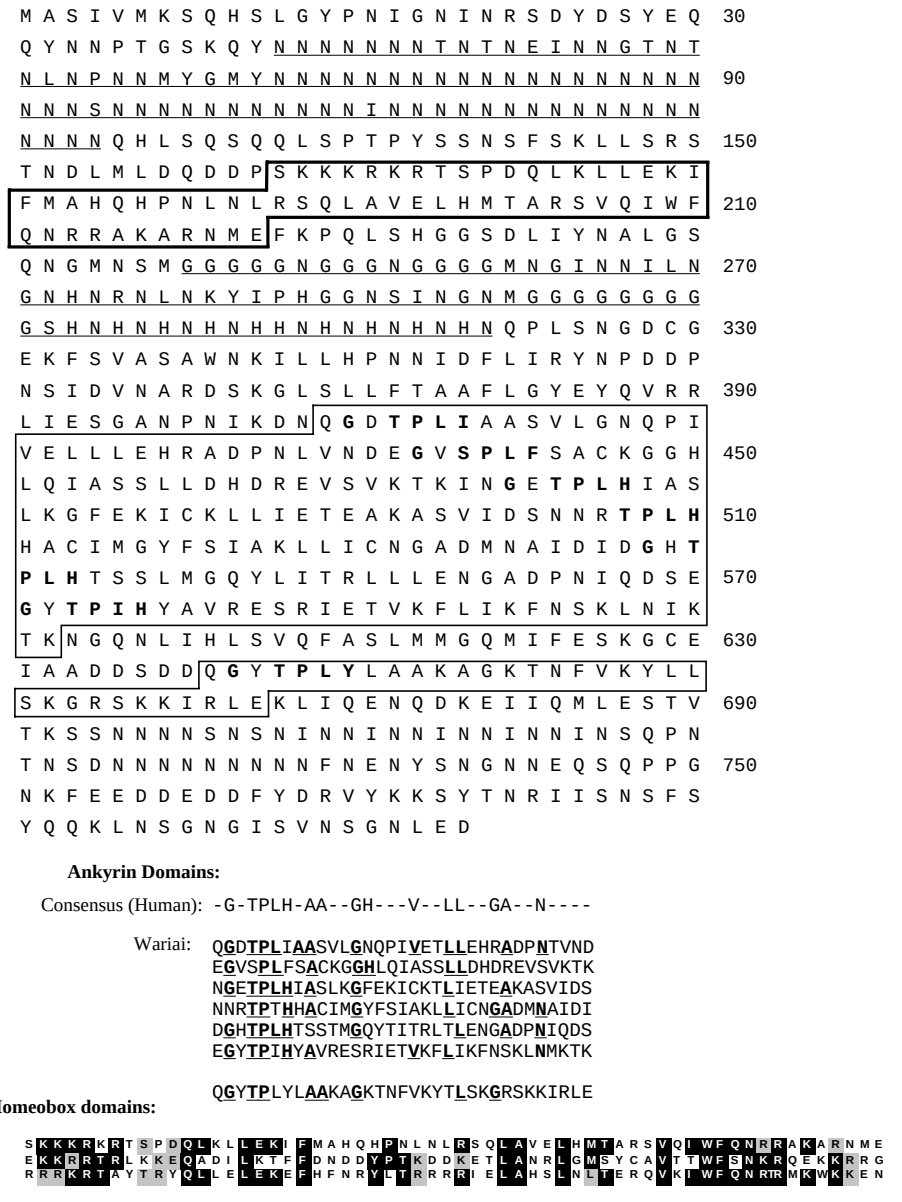


Fig. 1. Derived amino acid sequence of *Dictyostelium* homeobox-containing genes *Wri* and *DdHbx-2*. Amino acid sequence of *Wri* is shown. Homopolymer-rich domains are underlined. The 60 amino acid homeobox is boxed in a thick line. The ankyrin domains are boxed in a thinner line. The first 6 ankyrin domains are contiguous, while the seventh is separated. In addition, comparison of the *Wri* ankyrin domains with the consensus human ankyrin domain is shown. Amino acid sequence identities with the amino acids in the consensus ankyrin domain are in bold and underlined. Comparison between *Wri* and *DdHbx-2* homeobox domain and a consensus homeobox domain (Burglin, 1995) is shown.

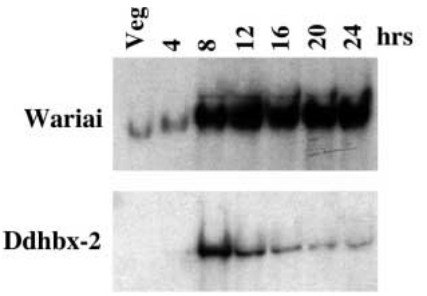


Fig. 2. Developmental RNA blot of *Wri* and *DdHbx-2*. RNA was isolated from cells at the indicated times in development and analyzed by RNA blot hybridization. Eight hours is the time of early mound formation and the time of the developmental switch between aggregation and cell-type differentiation. 16 hours is the approximate time of the slug stage, which is only very transient under these conditions. It is at this time that GBF-regulated genes are first induced.

the anterior of *wri* null slugs was not as cylindrical as that of wild-type slugs (Figs 4, 5). When *Dictyostelium* cells are grown on nutrient plates in association with a bacterial food source, clones originating from a single cell grow vegetatively, feeding on the bacteria, and form a 'plaque' in the bacterial lawn as the bacteria are eaten. As the bacteria are depleted, the cells starve and initiate multicellular development. The morphological phenotype of the *wri* null cells is more severe under these developmental conditions than when grown axenically and plated on non-nutrient buffered agar (Fig. 3C,E). Wild-type cells show a similar developmental morphology whether allowed to develop on nutrient or non-nutrient plates (Fig. 3A,B).

ddhbx-2 null cells, made and examined using the same approaches as for disruption of *Wri*, are morphologically indistinguishable from wild-type cells regardless of which genetic background and marker was used (data not shown). Double knockouts (*wri/ddhbx-2* null cells) were made in which the Bsr *Wri* knockout construct was used to disrupt the *Wariai* gene in *ddhbx-2* null cells created using *Thy1* selection. The *DdHbx-2* Bsr knockout construct was also used to disrupt the *DdHbx-2* gene in *wri* null cells made using the Thy1 marker. In this way, both reciprocal double knockout strains were obtained. Morphologically, the double knockout strains appeared similar to *wri* null cells, although they may have a slightly enhanced morphological phenotype (Fig. 3G-I). In addition, the phenotypes are slightly stronger than those of *wri* null cells (Fig. 2), with a greater fraction of the aggregates arresting at the mound stage and larger increase in the lower stalk region. All sets of experiments described below were done with both *wri* null strains and both *wri/ddhbx-2* double knockout strains. No differences were identified between the *wri* null strain made using either marker or between the reciprocal double knockout strains.

Wariai regulates the size of the pstO compartment

Spatial patterning of the cell types was examined in wild-type, *wri* null, *ddhbx-2* null and double knockout strains transformed

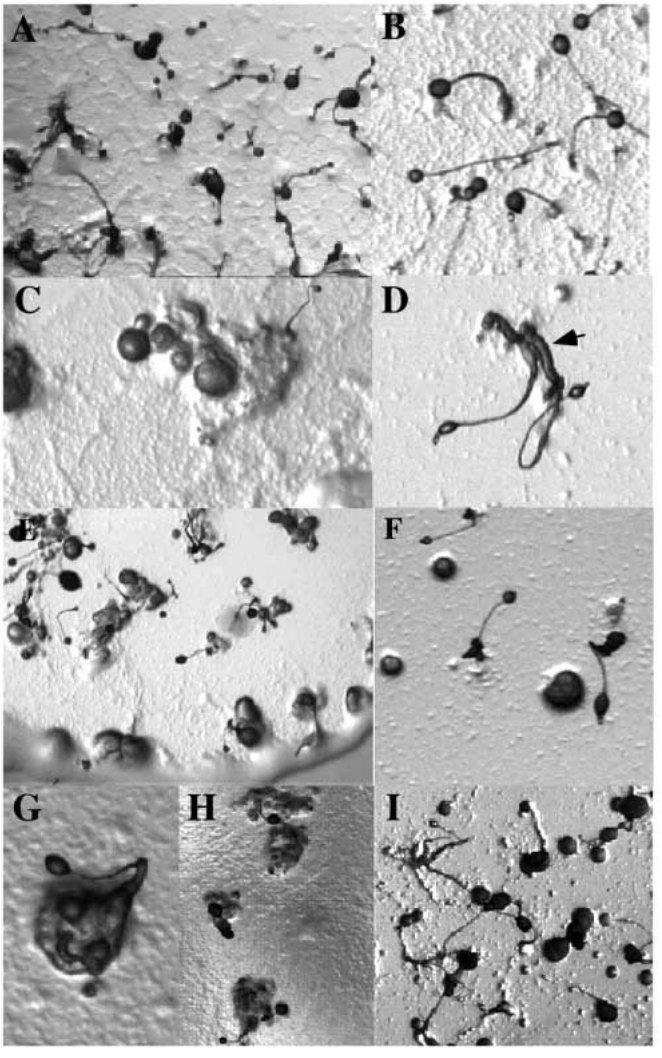
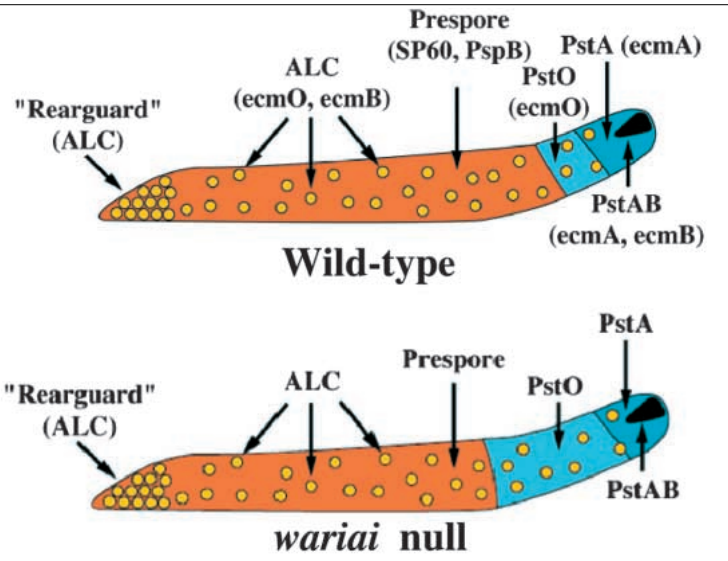


Fig. 3. Developmental morphologies of wild-type and *wri* and *wri/ddhbx-2* null strains. To examine morphological developmental defects, both strains were grown axenically, washed free of medium, and plated on non-nutrient, phosphate buffer-containing agar plates or grown in association with *Klebsiella aerogenes* bacteria on SM nutrient plates. In the latter case, after the bacteria are depleted, *Dictyostelium* cells starve and initiate development. Panels A, C, E, G, and F are cells grown in association with bacteria on SM nutrient plates. Panels B, D, F, and I are cells grown axenically and developed on non-nutrient agar plates. Panels A and B, wild-type cells; panels C, D, E, and F, *wri* null cells; panels G, H, and I, *wri/ddhbx-2* null cells. In Panel B, the overall view of wild-type fruiting bodies can be seen with a round sorus containing the spores on top of a thin stalk. In Panel D, the thickening of the basal disc (marked with an arrowhead) and lower stalk region of *wri* null strains can be observed. In addition, notice the smaller size of the sorus compared to the wild-type cells and general size of the aggregate. Numerous aggregates that arrest at the mound stage can be seen in *wri* null or *wri/ddhbx-2* (double knockout) null strains (Panels C-I). In Panel E, one can observe the edge of the growing *Dictyostelium* plaque in the bacterial lawn in the lower portion. That elevated edge and lower gray area represent the edge of the bacterial food source as the *Dictyostelium* cells move into a previously undigested area and digest the bacteria. Abnormal morphology of a *wri/ddhbx-2* double knockout strain grown on bacteria can be seen in Panels G and H. The *wariai* null obtained using the Thy1 marker is shown and was used for transformation of the *lac*Z constructs and histochemical staining (see below). The double knockout shown and used for the staining is a strain in which the *Wri* gene was disrupted with *Bsr* and *DdHbx-2* with *Thy1*.

Table 1. Comparison of average ratios of specific cell types



cell line	Marker	prestalk AO domain	prespore domain	post. (rearguard)
KAx-3 (WT)	SP60/lacZ	0.19 +/- 0.02	0.72 +/- 0.06	0.09 +/- 0.07
	PspB/lacZ	0.21 +/- 0.02	0.70+/- 0.06	0.09 +/-0.07
	ecmAO/lacZ	0.22 +/- 0.03	0.78 +/- 0.03	
	RasD/lacZ	0.22 +/- 0.02	0.78 +/- 0.02	
wri null	SP60/lacZ	0.31 +/- 0.04	0.58 +/- 0.04	0.11 +/- 0.04
	PspB/lacZ	0.31 +/- 0.06	0.54 +/- 0.05	0.14 +/- 0.06
	ecmAO/lacZ	0.35 +/- 0.02	0.65 +/- 0.02	
	RasD/lacZ	0.33 +/- 0.03	0.67 +/- 0.03	
double KO	SP60/lacZ	0.30 +/- 0.03	0.55 +/- 0.04	0.15 +/- 0.04
	PspB/lacZ	0.34 +/- 0.05	0.56 +/- 0.04	0.10 +/- 0.08
	ecmAO/lacZ	0.35 +/- 0.04	0.65 +/- 0.04	
	RasD/lacZ	0.36 +/- 0.03	0.64 +/- 0.03	
cell line	**Marker**	**prestalk A domain**	**prestalk O domain**	**ecmO staining***
KAx-3 (WT)	ecmA/lacZ	0.090 +/- 0.018		
	ecmO/lacZ	0.092 +/- 0.019	0.13 +/- 0.018	**0.11 +/- 0.01**
wri null	ecmA/lacZ	0.095 +/- 0.015		
	ecmO/lacZ	0.12 +/- 0.017	0.22 +/- 0.03	**0.26 +/- 0.02**
double KO	ecmA/lacZ	0.105 +/- 0.014		
	ecmO/lacZ	0.13 +/- 0.03	0.26 +/- 0.05	

Wild-type (KAx-3), *wri* null and *wri/ddhbx-2* (double knockout) null cells were stably transformed with the following *lac*Z reporter constructs: *SP60*, prespore-specific; *PspB*, prespore-specific; *ecmAO*, prestalk (pstAB, pstA and pstO)- and ALC-specific; *rasD*, prestalk- and ALC-enriched; *ecmA*, pstA-specific; *ecmO*, pstO-specific; ecmB, pstAB- and ALC-specific. Cells were developed on Millipore filters placed on top of Na/K phosphate plates and allowed to develop until the slug stage (Haberstroh and Firtel, 1990; Mann et al., 1994). Cells were then histochemically stained for β-gal activity. Both the stained and non-stained regions were measured from the anterior and normalized as a fraction of the length of the slug, with 0.0 being the very anterior tip and 1.0 being the very posterior of the slug. For the cells stained using the two prespore-specific reporters, *SP60/lac*Z and *PspB/lac*Z, the prestalk domain was quantitated as the region between the very anterior end of the slug and the start of the prespore staining domain. The prespore domain was the region that was stained. In many slugs, a posterior non-stained region was identified and designated as the 'rearguard.' This region has been shown to be enriched in anterior-like cells (ALCs) and to be periodically sloughed off during slug migration (see text). Because of the sloughing off during migration, this domain can be longer or shorter, depending on when during this process the slugs are fixed and stained. For *ecmAO/lac*Z- or *rasD/lac*Z-expressing cells, the prestalk domain was designated as the anterior darkly staining domain. The prespore domain was designated as the posterior unstained or less intensely stained portion of the slug and comprises both the prespore and rearguard regions. Depending upon the timing, the rearguard may or may not be present and, if present, is visible as a stained region at the very posterior of the slug using these markers. For the *ecmA* construct, the pstA domain was designated as the stained region. For *ecmO/lac*Z, the very anterior unstained region is designated pstA, the stained region as pstO, and the posterior as the prespore and rearguard. For measurement, slugs were picked at random with the only consideration being that the slugs do not have a major bend, or are S-shaped, which would preclude accurate length measurements of the various domains. Slugs chosen, for the most part, were straight. A minimum of 20 slugs were measured and the standard deviation calculated.

Wild-type and *wri*$^{-}$ slugs expressing *ecmO/lac*Z were also dissociated and the cells adhered to coverslips prior to staining for β-gal activity. Stained cells were then counted. Three separate, independent experiments were performed and >300 cells counted for each experiment. This column is labeled '**ecmO staining***.'

The organization of wild-type slugs and the expression pattern of the reports used in this study are shown as well as the spatial patterning in *wri* null cells. The gene organization of the cell-types within the slug is derived from Abe et al. (1994).

with *lac*Z reporter constructs in which β-gal is expressed from cell-type-specific promoters [*SP60* (prespore-specific), *ecmA* (pstA-specific), *ecmO* (pstO-specific), *ecmB* (pstAB and ALC) and *ecmAO* (expressed in pstAB, A and O cells and ALCs, with a stronger expression in pstA than pstO cells) (Early et al., 1993; Jermyn et al., 1989; Haberstroh and Firtel, 1990)]. In addition, we examined the spatial pattern of expression of the *rasD*/*lac*Z reporter, which shows a significantly stronger level

of expression in the anterior pstA/O/AB region and ALC population than in prespore cells (Esch and Firtel, 1991). A cartoon is presented in Table 1 that outlines the pattern of expression in the slug of the reporters that were used. Spatial patterning was also analyzed statistically by quantifying the anterior-posterior boundaries of the staining of individual reporters where 0.0 is the very anterior of the slug and 1.0 is the very posterior. Because cell-type proportioning is independent of the size of the slug (Firtel, 1995; Loomis, 1975), such measurements provide an accurate mechanism of quantitating the relative size of various cell-type compartments. The size independence of spatial patterning, which has been observed previously for wild-type organisms, was also observed in our mutant studies in which slugs of varying sizes showed the same spatial patterning.

Analysis of the prestalk- and prespore-specific *ecmAO* and *SP60* reporter staining shows that the anterior prestalk domain comprising the pstAB, A and O domains is significantly enlarged in the *wri* null and double knockout strains compared to that seen in wild-type cells and there is a concomitant decrease in the size of the prespore domain in the mutant strains (Fig. 4A; see Table 1 for quantitation). To determine which prestalk domains were affected in *wri* null cells, we used the pstA-(*ecmA/lac*Z) and pstO-specific (*ecmO/lac*Z) reporters (Fig. 4B). Examination of stained slugs and a quantitative analysis of the data (Table 1) show that there is an approximate doubling (see below) in size of the pstO compartment that is found at the coordinates ~0.1-0.22 units along the anterior-posterior axis in wild-type KAx-3 cells. This increases to coordinates ~0.1-0.35 in *wri* null cells, with a decrease in the size of the posterior prespore/rearguard domains. In contrast, the pstA (*ecmA*-expressing) and pstAB (anterior *ecmB*-expressing) compartments are unchanged (Fig. 4B; data not shown for the *ecmB* marker that is specific for the anterior pstAB domain in the slug). We also notice that, in *wri*$^{-}$*/SP60/lac*Z slugs, there are some prespore cells (cell expressing β-gal) in the enlarged pstO domain that are not observed in wild-type slugs. The results obtained from direct measurements of the various domains and indirect subtractive measurements of the stained slugs are all consistent (Table 1). No difference was observed between measurements made in which the *Wariai* gene was disrupted by the *Thy1* marker and using the Bsr selection (data not shown).

Developmental RNA blots were performed to examine the timing and level of expression of *ecmAO, ecmB* and *SP60* in *wri* null and wild-type cells. No reproducible, significant differences were observed between the mutant and wild-type

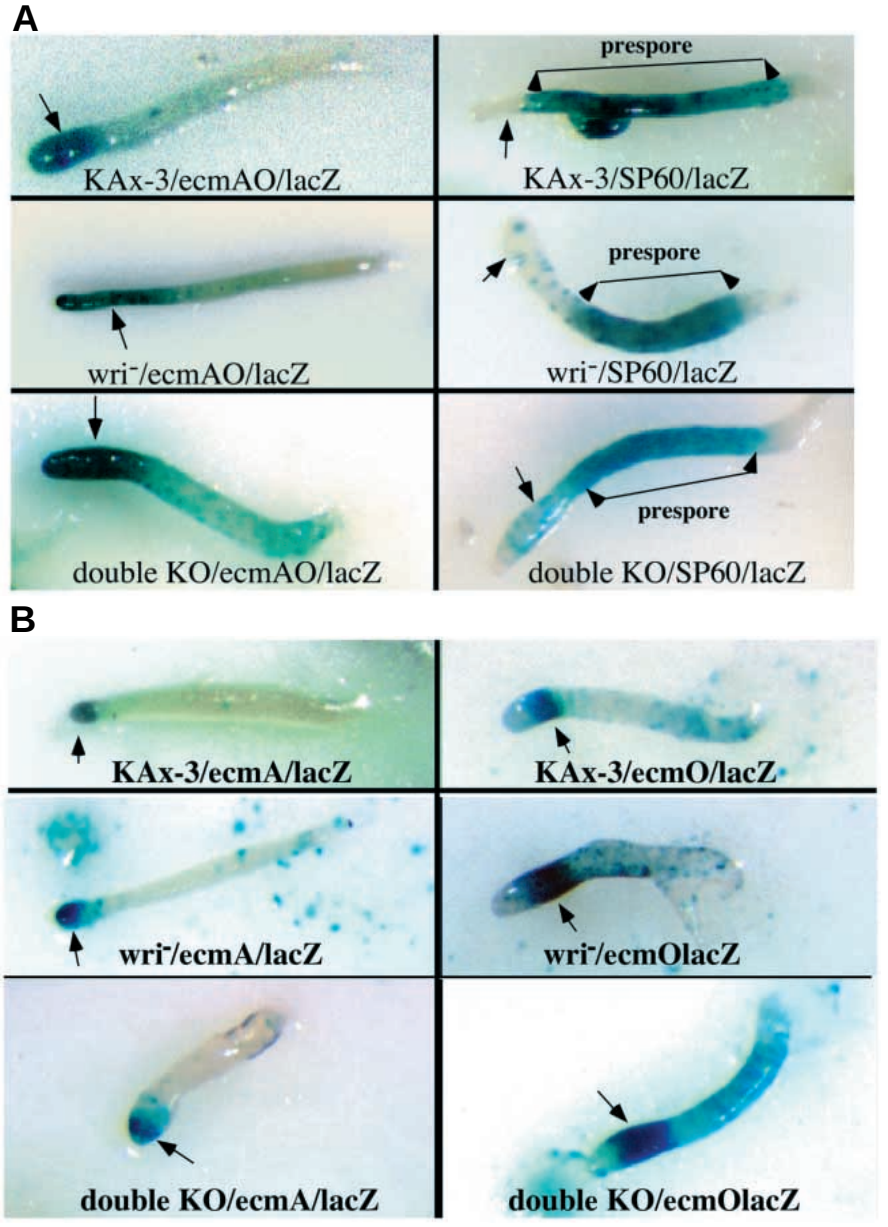


Fig. 4. β-gal histochemical analysis of spatial patterning of prestalk and prespore cells in wild-type and mutant strains. A. Cells were transformed with either the complete *ecmAO/lac*Z promoter construct (left side) or the prespore-specific reporter *SP60/lac*Z (right side). *ecmAO* is expressed in the anterior prespore-prestalk domain including pstAB, pstA, and pstO compartments. The arrowhead points to the anterior prestalk region. In *wri* null (*wri*$^{-}$ and *wri/ddhbx-2* double knockout) *wri*$^{-}$*/2*$^{-}$ strains, some *SP60/lac*Z-expressing cells can be seen in the prestalk domain. This is greater in the double knockout strain. The very posterior region that is unstained in slugs expressing *SP60/lac*Z is the posterior 'rearguard' region. B. Cells were transformed with either the *ecmA/lac*Z (pstA-specific; left side) or *ecmO/lac*Z (pstO-specific; right side). The arrowhead points to either the pstA (left side) or pstO (right side). Note the unstained pstA domain anterior to the pstO domain in the right-hand panels. This is unchanged in size in the three strains, while the pstO domain is increased in the mutant strains. All photographs are at the same magnification. Slugs are not all the same size. No difference in spatial patterning was observed between large and small slugs.

cells, presumably because northern blots are not sufficiently quantitative and pstO cells only weakly express the entire *ecmAO* promoter compared to pstA cells (Early et al., 1993), which are unaffected in *wri* null cells (data not shown). Similar studies performed with *ddhbx-2* null strains showed no difference from the results observed in wild-type strains (data not shown). When these studies were done in the *wri/ddhbx-2* double knockout cells, the results were similar to those in *wri* null cells except that the increase in the prespore O domain was slightly larger (Fig. 4; Table 1). We also observe a greater number of *SP60/lac*Z-expressing cells in the pstO domain than are observed for the *wri* null cells (Fig. 4, part I, *SP60/lac*Z panels).

The increase in the number of the *ecmO/lac*Z-staining cells was quantified counting the number of *ecmO/lac*Z-staining cells in *wri* null and wild-type slugs after they were dissociated into single cells and the cells histochemically stained. This analysis showed 11± 1% of the wild-type and 26 ± 2% of the *wri* null cells stained, indicating there was an ~2.5-fold increase in the number of *ecmO*-expressing cells in the mutant. The *ecmO/lac*Z-staining cells are expected to be a combination of pstO cells and ALCs.

As a further method to examine the relative size of prestalk and prespore domains, we employed double-label reporter analysis using *ecmAO/lac*Z and *SP60*/β-glucuronidase (GUS) reporters. Clonal isolates of strains expressing both markers were obtained and histochemically stained for β-galactosidase and β-glucuronidase activity (Haberstroh and Firtel, 1990; Jermyn and Williams, 1991; Early et al., 1993; Mann et al., 1994). The results presented in Fig. 5 clearly show a significant increase in the prestalk (magenta-stained) domain (*ecmAO/lac*Z) and a decrease in the prespore (blue) domain (*SP60/GUS*) in *wri* and *wri/ddhbx-2* null strains when compared to wild-type cells. The increase in the pstAO domain in these experiments using the *ecmAO/lac*Z reporter and magenta-gal substrate is greater than that observed using X-gal, although the constructs used are identical (Jermyn and Williams, 1991). This may be due to the staining properties and/or differences in the hydrolysis rates of the two substrates by β-gal. Nonetheless, using either marker, there is a significant increase in the pstAO domain in *wri* null cells. The double-staining results also suggest little overlap, if any, between the prestalk and prespore domains.

The spatial pattern of expression of *ecmAO, ecmO* and *SP60* was also examined in mature fruiting bodies. With either the *ecmAO/lac*Z or *ecmO/lac*Z reporter, *wri* null cells showed a more intense staining in the basal disk and upper and lower cups, regions that are derived from the ALC populations (data not shown). Prespore marker (*SP60/lac*Z) staining of the spore mass showed a narrower band of staining in the null cells compared to wild-type cells (data not shown). These data are consistent with an increase in the pstO/ALC population in the mutant strains when compared to wild-type cells.

The homeobox and ankyrin domains of *Wariai* are required for function

Neutral red is a vital dye that preferentially stains an acidic vacuole found in prestalk cells and anterior-like cells (ALCs) but is absent in prespore cells in the slug (Loomis, 1982; Sternfeld and David, 1982). Neutral red has been used as a prestalk and anterior-like cell marker and a mechanism to quantitate sizes of prestalk compartments and localize and monitor movement of ALCs within the prespore region (Abe et al., 1994; Dormann et al., 1996; Hadwiger et al., 1994; Jermyn and Williams, 1991; Siegert and Weijer, 1992, 1995). In confirmation of the *lac*Z reporter analysis, neutral red staining shows that the anterior, neutral red staining region is significantly enlarged in *wri* null cells compared to wild-type cells (Fig. 6). Transformation of the *wri* null cells with the *Wariai* expression vector restores the wild-type neutral red staining pattern (Fig. 6) and complements the *wri* null morphological defects (data not shown). To determine whether both the homeobox and the ankyrin repeats are required, constructs carrying an in-frame deletion of either part of the Hbx domain (*Act15-WriΔHbx*) or the ankyrin repeats (*Act15-WriΔAnk*) were transformed into *wri* null cells. As shown, neither deletion construct was able to restore neutral red staining (Fig. 6) nor morphological defects observed in *wri* null cells (data not shown). These results indicate that both the homeobox domain and the ankyrin repeats are required for *Wariai*. Cells overexpressing the *Wariai* coding region showed no morphological defect or change in neutral red staining (data not shown).

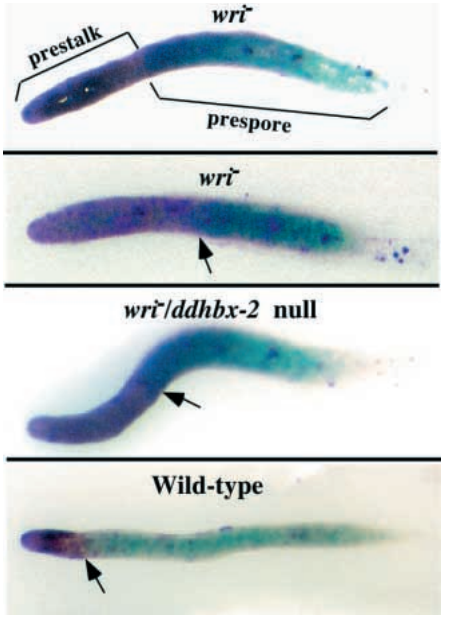


Fig. 5. Double staining of prestalk and prespore domains. Wild-type and null strains were stably cotransformed with *ecmAO/lac*Z and *SP60/GUS* and stained at the slug stage for expression of both markers using magenta-gal and X-Glc as a substrate for β-gal and β-gluc, respectively. The arrowhead points to the demarcation between the anterior prestalk (magenta) and posterior prespore (blue) domains.

Expression of *Wariai* is prestalk-specific during multicellular development

We used a novel approach to clone the *Wariai* promoter. To accomplish this, we integrated, by homologous recombination, a pUC-based *E. coli* plasmid into the *Wri* gene (see Materials and Methods). Homologous recombination into the *Wri* locus was confirmed by Southern blot analysis and the strains exhibited the same morphological defect as the other *wri*

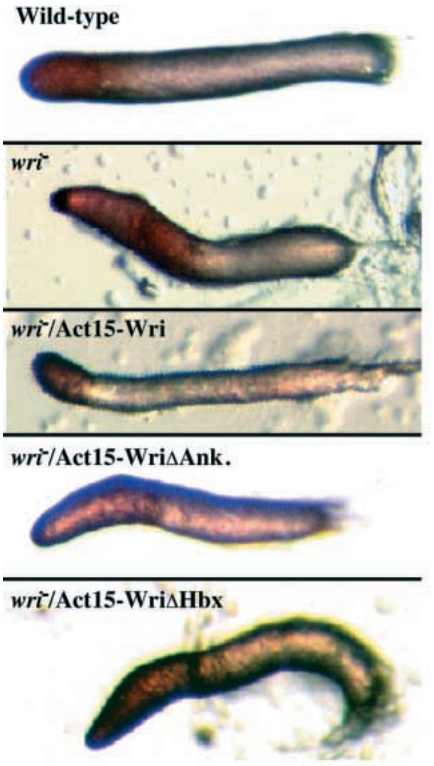


Fig. 6. Neutral red identification of pstA domains in wild-type and mutant cells. Wild-type cells, *wri* null cells, *wri* null cells complemented with *Act15-Wri* (expressing the complete *Wariai* ORF), or *Act15-WriΔAnk*, or *Act15-WriΔHbx*-expressing proteins with in-frame deletions of either the *Wariai* ankyrin or *Wariai* homeobox domains, respectively. Neutral red stains the anterior prestalk domain more intensely than the prespore domain. Prespore staining is due in part to ALCs located in this domain that also have acidic vacuoles that stain with neutral red.

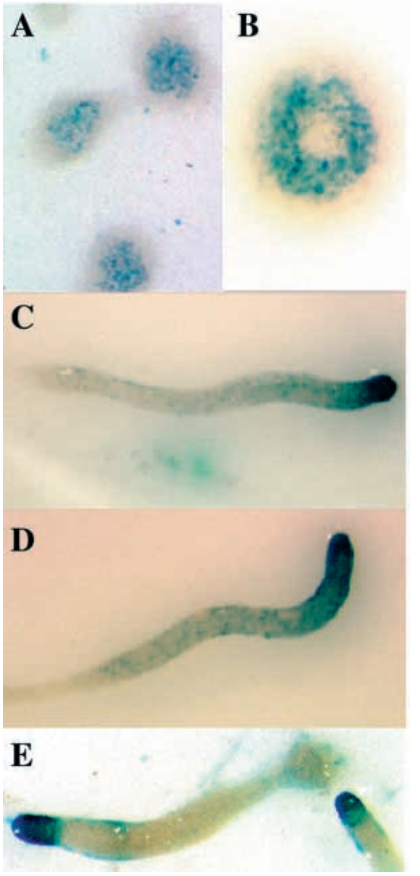


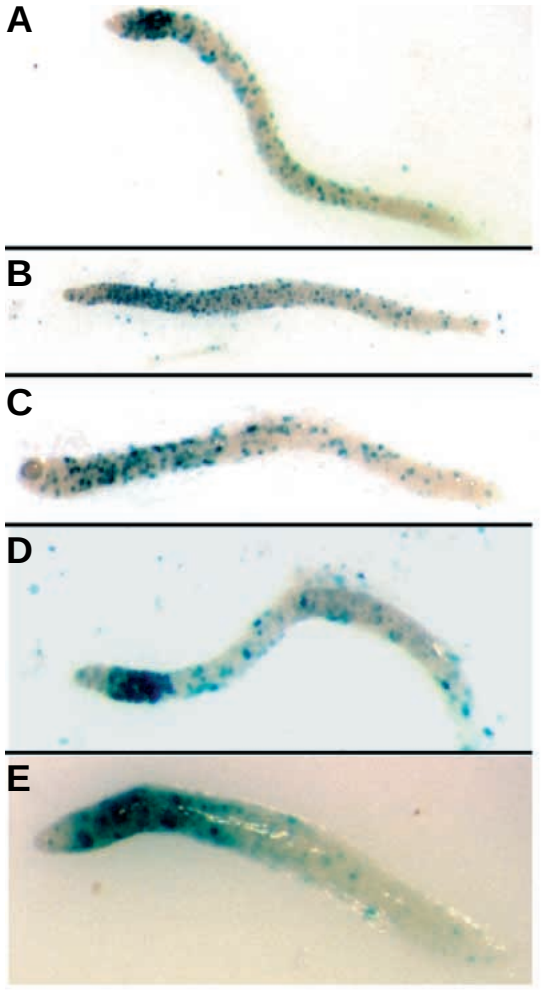
Fig. 7. Spatial pattern of expression of *Wri*. Wild-type or *wri* null cells were transformed with *Wri* promoter in-frame with *lac*Z (see Materials and Methods). Cells were histochemically stained at the mound and slug stages for β-gal activity. Wild-type cells: panels A and B, mound stages at different times; panels C and D, slugs. The staining observed in panel B is at an earlier stage than seen in panel A. *wri* null cells: slug stage, panel E.

knockouts (data not shown). The 5′ flanking region of *Wri* was isolated by linearizing genomic DNA 2.5 kb upstream from the start codon and at a designated site within the inserted vector, ligating the DNA and cloning the recircularized vector carrying the 5′ flanking region into *E. coli*. This cloned 5′ region was fused in-frame to the β-gal reporter and transformed into wild-type and *wri* null cells and stably transformed clones were isolated. As shown in Fig. 7C,D, the *lac*Z expression pattern in wild-type slugs is prestalk-specific with very strong staining in the pstA and some scattered throughout the posterior, which probably represents expression in anterior-like cells. No definitive staining of the pstO compartment of migrating slugs is observed. In the developing mound, staining is first seen as a ring of cells (Fig. 7B) that then moves toward the developing tip and is observed as a centrally localized group of cells near the top of the mound (Fig. 7A). This staining pattern is remarkably similar to that of pstA cells, as previously described (Early et al., 1995). In the mature fruiting body, expression is seen in the stalk and upper and lower cups of the sorus, consistent with a pstA/O-like expression pattern (data not shown). When the staining pattern of *Wri/lac*Z was examined in *wri* null cells, the staining pattern was unchanged, remaining highly localized to the anterior pstA compartment (Fig. 7E). No expansion of the staining pattern was observed as is seen with *ecmAO/lac*Z or *ecmO/lac*Z. This is consistent with *Wri* expression in the anterior of the slug being restricted to the pstA region.

Expression of the *Wri* construct from the cloned *Wri* or the ecmA, pstA-specific, promoter complemented the *wri* null phenotype as determined by quantitation of the size of the prestalk zone by neutral red staining as described above (data not shown). However, expression of *Wri* from either the pstO-specific *ecmO* or the prespore-specific *SP60* promoter did not complement the *wri* null phenotype (data not shown).

Wariai functions to regulate the size of the pstO compartment

From the *Wri/lac*Z staining, *Wri* expression appears localized to pstA and ALCs even though the *wri* null mutation alters the size of the pstO compartment, suggesting that *Wri* may function non-autonomously. To further examine a possible non-autonomous function for *Wri*, we created chimeras composed of *wri* null and wild-type cells, with one of the strains expressing the *ecmO/lac*Z reporter to mark pstO cells. Similar experiments were performed with the prestalk marker *SP60/lac*Z. In these experiments, the cells carrying the reporter



Figs 8, 9. Spatial localization of pstO cells in wild-type:*wariai* null strain chimeras. Chimeric organisms were formed by mixing wild-type and *wri* null cells and allowing them to coaggregate. Of the chimeras, 1 part of either wild-type or mutant cells expressing the *ecmO/lac*Z reporter (pstO-specific) was mixed with 3 parts of the reciprocal strain (*wri* null or wild-type cells) that did not carry the marker. Slugs were allowed to form and histochemically stained for β-gal activity to localize the *ecmO*-expressing cells in the labeled strain. As a control, 1 part of labeled *wri* null cells was mixed with 3 parts of unlabeled *wri* null cells or 1 part labeled wild-type cells was mixed with 3 parts unlabeled wild-type cells and stained.
Fig. 8. (A) Wild-type cells expressing *ecmO/lac*Z mixed with 3 parts unlabeled wild-type cells; (B,C) wild-type cells expressing *ecmO/lac*Z mixed with 3 parts *wri* null cells; (D)*wri* null cells expressing *ecmO/lac*Z mixed with 3 parts wild-type cells; (E) *wri* null cells labeled with *ecmO/lac*Z mixed with 3 parts unlabeled *wri* null cells. Using a smaller fraction of *lac*Z-marked cells resulted in too few stained cells to properly identify the spatial pattern.

were mixed with three parts of unlabeled cells. In some experiments, a fraction of the cells carrying the *lac*Z reporter were also labeled with the vital fluorescent stain Cell-Tracker Green (CMFDA, Molecular Probes) as a 'genetic' tag, which allows us to determine the general distribution of this strain within the slug. As shown in Fig. 8B, mutant and wild-type cells distributed uniformly throughout the slug. As expected, homologous mixtures (one part *ecmO/lacZ-wri* null cells mixed with three parts unlabeled *wri* null cells or one part *ecmO/lac*Z wild-type cells mixed with three parts unlabeled wild-type cells) showed the same spatial distribution of *ecmO/lac*Z-expressing cells as described above when the *wri* null and wild-type strains were examined individually, except that the density of the label is reduced due to the presence of fewer *ecmO/lac*Z-expressing cells (compare Fig. 8D,E to Fig. 4B). When one part *ecmO/lacZ-wri* null cells was mixed with three parts unlabeled wild-type cells, the size of the pstO domain was slightly larger than that of a wild-type:wild-type homologous chimera (compare Fig. 8A-D). However, when one part *ecmO/lac*Z-wild-type cells was mixed with three parts unlabeled *wri* null, the labeled wild-type cells (Fig. 8B,C) showed a significantly broader distribution than that observed with the control homologous wild-type:wild-type mixture in the reciprocal mix, which was similar to the homologous wri^{-}/wri^{-} mixture. Thus, in a slug where 75% of the cells are unlabeled *wri* null cells, there is a significant expansion of the region occupied by the wild-type pstO cells, which is consistent with a significantly enlarged pstO compartment.

Experiments using the *SP60/lac*Z reporter show a similar pattern, with the majority strain in the chimera dictating the size of the prestalk and prespore compartments (Fig. 9). In the chimera containing one part wri^{-}*/SP60/lac*Z and three parts wild-type cells, the size of the prestalk domain was only slightly larger than that observed for wild-type strains and the prespore domain was slightly smaller (Fig. 9B). In the reciprocal chimera (75% of the cells are unlabeled *wri* null cells), there is a significant reduction of the region occupied by wild-type prespore cells (*SP60/lac*Z-expressing cells; Fig. 9D), which is consistent with the above model.

DISCUSSION

Wariai regulates the size of the pstO compartment

Wariai and *DdHbx-2* are the first homeobox-containing genes that have been identified in *Dictyostelium*. Disruption of *Wri* leads to a more than doubling of *ecmO/lac*Z-expressing cells and the pstO compartment and no detectable change in the relative size of the more anterior pstA and pstAB compartments. Furthermore, we show that both the homeobox domain and the ankyrin repeats are required for *Wri* function. As there is recent evidence that there is no cell division during morphogenesis (Shaulsky and Loomis, 1995) in *Dictyostelium* and spatial patterning is independent of the size of the organism, this increase in the size of the pstO compartment is not simply due to a mitotic doubling of the pstO cells. Our data suggest that there is a concomitant decrease in the number of prespore cells, it is probable that the increase in the pstO cells is at the expense of the prespore cells. As we discuss below, we propose that this might be the result of a conversion of prespore cells or cells destined to become prespore cells to pstO cells. While it is difficult to determine quantitatively if there is an increase in the ALC population, staining with an ALC marker (*Gα4/lac*Z reporter, Hadwiger et al., 1994) shows no clear differences between *wri* null and wild-type cells (ZH and RAF, unpub. obser.).

Our analysis suggests that disruption of *Wri* does not affect the ability of any known cell type to differentiate but leads to a shift in the equilibrium of prespore and pstO populations. It is possible that *Wri* regulates an interconversion of pstO and

prespore cells via transdifferentiation, which may play an important role in regulating the maintenance of cell-type proportioning. Two alternate models are possible. The first is that *Wri* does not affect the initial patterning but affects cell-type homeostasis, which leads to an increase in pstO cells and a decrease in prespore cells. In this model, *Wri* would not necessarily alter the initial spatial patterning of the prespore, pstA and pstO cell types, which are thought to derive from distinct subpopulations of cells within the developing mound (Early et al., 1995). The second is that *Wri* functions to control the sensitivity of cells to a morphogen such as DIF and effects the relative number of cells that become pstO cells. In this scenario, pstO cells would be induced at lower DIF concentrations and/or prespore cells would be more sensitive to DIF, resulting in a decrease in prespore cell differentiation. While we observed no differences in the initial number of *ecmO/lac*Z-staining (pstO) cells in the forming mound of *wri* null and wild-type strains (data not shown) in support of the first model, prestalk cells are still differentiating as the mound is forming, making it difficult to accurately quantitate the number of cells that will initially become pstO cells. We also identified a second homeobox gene, *Ddhbx-2*, that may potentiate the function of *Wri*. Both genes are developmentally regulated and induced by cAMP as the mound forms. While transdifferentiation is known to occur in migrating slugs and if slugs are dissected, it is possible that other mechanisms may also control cell-type interconversion and *Wri* may regulate these processes.

Wariai may regulate cell-type homeostasis in the slug by a cell non-autonomous pathway

Wariai shows a pstA- and ALC-specific spatial pattern of expression during multicellular development, with expression being highest in pstA and little, if any, expression in pstO cells. Moreover, expression of *Wri* protein from a pstO-specific promoter construct does not complement the *wri* null phenotype, suggesting the regulation by *Wri* on patterning is cell non-autonomous. The doubling of the size of the pstO domain in *wri* null cells suggests that *Wri* functions genetically to negatively regulate a signaling pathway controlling the size of the pstO compartment and is required for properly specifying the posterior boundary of this domain. We expect that *Wri* encodes a transcription factor that may function genetically as a repressor for a signaling pathway that is involved in negatively regulating prestalk cell specification. This is consistent with our observation of wild-type pstO cell distribution in chimeras containing a predominance of unlabeled *wri* null cells in which there is a significant increase in the size of the domain occupied by wild-type pstO (*ecmO*-staining) cells. These data are also consistent with an enlarged pstO compartment and a cell non-autonomous function for *Wri* in regulating patterning. Such a possible non-autonomous function of regulating spatial patterning by a homeobox-containing gene is quite distinct from that seen in metazoans where the pathways regulated by Hbx-containing genes are predominantly cell-autonomous (McGinnis and Krumlauf, 1992).

Approximately 30% of the *wri* aggregates arrest at the mound stage and do not form tips, a percentage that is higher in *wri*$^{-}$*/ddhbx-2* null cells. Tips are formed through the chemotactic migration of prestalk cells to the apex of the mound (see Introduction). As *Wri* is expressed in pstA, it is possible that *Wariai* may have a cell autonomous function to control tip formation, with null cells being less efficient in tip formation. It is possible that *Wri*'s regulation of tip formation and the pstO compartment are distinct functions of the gene and that *Wri* controls two very different pathways.

Wariai: an ancient regulator of anterior-posterior axis formation?

It is interesting to speculate on the possible evolutionary significance of the role of *Wariai*. In metazoans, it is known that some homeobox-containing genes regulate cell-type patterning and are involved in controlling anterior-posterior axis formation and determination. The *Dictyostelium* slug is a relatively simple and ancient multicellular organism with only a few cell types but a clear anterior-posterior axis. This forms after the cells sort

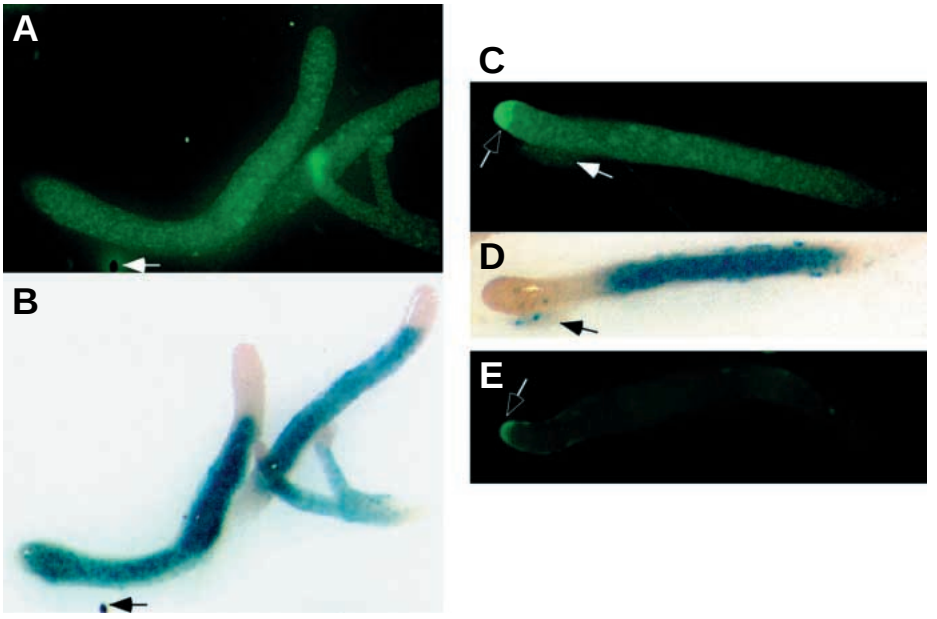


Fig. 9. (A,B) *wri* null cells expressing *SP60/lac*Z mixed with 3 parts unlabeled wild-type cells. Twenty percent of the *wri* null cells were labeled with the vital fluorescent dye Cell-Tracker Green (CMFDA, Molecular Probes). (A) The distribution of the labeled cells. (B) The distribution of the β-gal staining. (C,D) Wild-type null cells expressing *SP60/lac*Z mixed with 3 parts unlabeled *wri* null cells. 20% of the wild-type cells were labeled with the vital fluorescent dye Cell-Tracker Green (CMFDA, Molecular Probes). C shows the distribution of the labeled cells. (D) The distribution of the β-gal staining. (E) The background fluorescence of an unlabelled slug. Note the autofluorescence in the very anterior margin of the slug (open arrowhead). This autofluorescence is also seen at the tip of the slug in C. To identify the same slugs from in the photographs of the fluorescence images to view after staining, identification marks were used. These are marked with the solid arrowheads.

during tip formation and this anterior-posterior pattern can first be distinguished in the first finger stage. It is possible that the role of homeobox-containing genes in the control of the anterior-posterior axis is sufficiently ancient to include cell-type proportioning in *Dictyostelium*. In *Dictyostelium*, this may be regulated by changing the proportioning mechanism after differentiation of the individual cell types, possibly via the regulation of transdifferentiation or another process, in contrast to mechanisms that may be used in metazoans, which, in general, have more determinative cell fate decisions. The identification of genes directly regulated by *Wariai* should help elucidate the mechanism by which this gene controls spatial patterning.

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