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Changes in cell proliferation and cell survival are thought to be major fundamental causes of cancers. The *Drosophila* eye is a precise structure generated in part by apoptosis of excess cells during development. We identified a mutation named *pineapple eye* (*pie* ) that has too few cells in the retina. Cell proliferation is normal in *pie* mutants but excess apoptosis occurs. Multiple independent *pie* mutations have been collected. Amorphic mutations affect cell survival in many developing tissues causing developmental delay and death. We identified and characterized the *pie* gene. The *pie* gene is predicted to encode a novel 582 amino acid protein, perhaps interacting with molybdopterin. It is possible that the *pie* gene encodes a novel enzyme protecting against cell death during growth and development.

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## Table of Contents

## Introduction

Changes in cell proliferation and cell survival are thought to be major fundamental causes of cancers. Cell proliferation and cell survival are normally important in development and morphogenesis, but little is known about how their regulation in vivo. Although intracellular mechanisms of growth and survival have been studied extensively in tissue culture cells, these cells do not normally show regulated growth and morphogenesis. Regulation of these processes must be studied in vivo. The fruitfly Drosophila melanogaster provides a multicellular model organism suitable for genetic identification of growth and morphogenesis through study of mutations. We have isolated mutations that affect cell number in the developing Drosophila eye. These identify a candidate gene for growth regulation in vivo.

We performed mutations to identify mutations affecting Drosophila eye development(1). A recessive mutation mapping at 43 centimorgans along the second chromosome (cytological position 32A) has "rough" eyes and was named *pineapple eye* (*pie* ). In *pie* mutants the retina has too few cells, because of excess cell death during eye development. Therefore the *pie* gene is a candidate to encode a component of a survival signal. We proposed to clone the *pie* gene and characterize the role of its product. In previous annual reports, I described genetic and molecular characterization of the *pie* gene region, culminating in the definition of a ~120 kb interval within which the *pie* gene must reside, the mapping of an inversion breakpoint that was shown to disrupt *pie* gene function, the identification of the *pie* gene and prediction that *pie* encodes a novel 582 amino acid protein product.

## Results

### Technical objective 1. Characterization of chromosomal region 32A

Early on we discovered that initial mapping of the *pie* mutation to chromosome 32A was incorrect, and reassigned the gene to chromosome bands 31E-F. 66,500 flies derived from X-irradiated germ cells (4000rads) were screened for rearrangements and for failure to complement the original $pie^{EB3}$ allele. Several individuals appearing to carry newly-induced *pie* mutations were identified , but in no case did such individuals breed successfully, and the putative new mutations could not be recovered.

In an alternative approach made possible by the relocalization of *pie* to 31E/F, we determined that the $pie^{EB3}$/deficiency phenotype was semilethal. Those adults that do survive are extremely sickly; the females are invariably sterile and the males breed very poorly.

These findings rendered our original scheme for isolating new *pie* mutations problematic. On the other hand, it raised the possibility that *pie* mutations could be identified through screening for lethal mutations. We obtained all the published lethal strains for the 31E-F region (2)and found that a previously-identified, but uncharacterized, lethal locus called $l(2)31E_k$ was allelic to *pie*. One allele, $l(2)31E_k^{G2\text{-}4}$, had been induced after X-ray mutagenesis(2). We found that $l(2)31E_k^{G2\text{-}4}$ was associated with a small cytologically-visible inversion between chromosome bands 31E and 32F. We had thus achieved Technical Objective 1 of characterizing

the *pie* gene region, by a modified route.

Technical Objective 2. Identification of minimally affected region

Genomic clones from the 31E region were obtained from clones collected by the European component of the Drosophila Genome Project(3). We established a contig of three overlapping cosmid clones that extended from the Sequence-Tagged-Site corresponding to the *daughterless* gene, which we determined to map distal to the *pie* locus, to the end of the *Df(2L)J77* deficiency, determined to map proximal to the *pie* locus. The *pie* genomic DNA had to lie within this interval of ~120 kb.Thus our original plan to define the *pie* locus precisely through characterization of chromosome rearrangement alleles was achieved through a slightly different route than originally proposed (Figure 1).

Genomic southern blotting from the $l(2)31E_k^{G2\text{-}4}$ mutation identified a breakpoint at position +0 kb which was not present in the progenitor strain from which $l(2)31E_k^{G2\text{-}4}$ had been isolated. Further molecular and cytological analysis showed that this +0 kb breakpoint corresponded to the proximal breakpoint of the 31E-32F inversion, identifying a specific region essential for normal *pie* gene function (Figure 1). This represented the achievement of Technical Objective 2.

Technical Objective 3. Isolation of the *pie* gene

Northern blotting and cDNA analyses identified three transcription units in the region defined as important for *pie* gene function (Figure 2). Full length cDNAs were isolated and their sequences determined. One transcript was homologous to human Replication Factor C (Genbank, Accession Number AF247494). One was a new cytokinesin-like gene (Genbank, Accession Number AF247500). The third was a novel open reading frame, apparently unrelated to any sequences from any organism in databases at that time (Genbank Accession Number 247501). Genomic DNA corresponding to two chemically induced *pie* mutations, $pie^{EB3}$ and $pie^{E1\text{-}16}$ , was PCR amplified, and sequenced, along with control DNA from the respective progenitor strains. For each mutation a single base substitution was identified compared to the control sequences, in both cases within the coding region for the novel third gene. No changes were observed in the coding regions for the Replication Factor C or cytokinesin-like genes. In $pie^{EB3}$ , residue 393 of the novel protein was replaced by a stop codon. In $pie^{E1\text{-}16}$, a frameshift in codon 205 predicted premature truncation after amino acid 220. Both substitutions were consistent with ethane-methyl sulfonate mutagenesis, and each predicted truncation of this open reading frame. These findings identified novel gene as the *pie* gene (Figure 2).

The *pie* gene is predicted to encode a 582 amino-acid protein (Figure 3). The sequence lacks predicted transmembrane, nuclear or mitochondrial import sequences and might encode a cytoplasmic protein.

The predicted sequence is notably acidic and cysteine-rich. It contains two major distinct regions differing in amino acid composition (Figure 3). Amino-acids 1-281 are cysteine rich (28 cysteines in this region). The pattern of cysteines does not correspond to that of known cysteine-based secondary structures, such as Ig-domains or EGF-repeats. Sequence databases contain three human and one additional *Drosophila* sequences that share significant similarity and Cys structure

with this domain of the *pie* gene, but these sequences are themselves of unknown function (Accession Numbers: AK000340; AB037754; AE002611; AL137671). Amino acids 290-510 are notably acidic and proline-rich. There are 29 prolines and 33 acidic glutamate or aspartate residues in this region. There are no other sequences with significant similarity to this domain.

The amino-terminal cysteine-rich portion of the predicted PIE protein contains the conserved hallmark of a molybdopterin-binding domain(4). Other molybdopterin-incorporating proteins that contain this consensus include aldehyde oxidase, nitrate reductase, and sulfite oxidase(4). This feature suggests that *pie* might encode an enzyme, such as a novel oxidase or reductase.

Technical Objective 4. Investigation of survival signal

In order to probe the role of *pie* in cell survival, we constructed plasmids for GAL4-targeted expression of the *pie* cDNA, and introduced these plasmids into the *Drosophila* germline by P element-mediated germline transformation. Such transgenic flies have been used to target ectopic *pie* expression to the eye and wing, tissues where cell death plays roles in normal morphogenesis. We have observed no effect on development or cell death after targeted *pie* expression. This suggests that *pie* encodes an essential component for cell survival, but may not be sufficient to promote cell survival in cells fated to die. The results do not suggest that restricted expression of *pie* is responsible for the pattern of survival and death during normal development of the eye or wing, or that cessation of *pie* expression is a trigger for cell death in normal development.

In order to elucidate the biochemical role of PIE in cell survival, we sought to raise antisera specific for the PIE protein and determine its pattern of expression in developing tissues, and location within the cell. We sought to express PIE protein in bacterial cells and purify it for use as antigen. Two expression systems have been used: T7 polymerase expression of histidine-tagged proteins and purification by Nickel-affinity; IPTG induction of Lac-promoter regulated expression of Glutathione S-Transferase (GST) fusion proteins.

Significant technical problems have been encountered. We have largely been unable to express either kind of protein in bacteria, even using a variety of different subportions of the PIE protein. Reasoning that the novel Cys-rich and Pro-rich portions of PIE might somehow prevent bacterial expression, we made constructs designed to express only the very carboxyl-terminal 93 amino-acids, which do not exhibit these sequence features. In addition this peptide Asn490-Ser582 contains a segment (Phe531-Asp540) predicted to be antigenic. We succeeded in expressing GST-PIE(F531-S582) in bacteria (although the corresponding His-tagged T7 protein was not expressed). GST-PIE(F531-S582) was insoluble and could not be purified by Glutathione affinity, however.

GST-PIE(F531-S582) has been purified by preparative SDS-gel electrophoresis and electroelution. The fusion protein has been used to immunize mice from two strains (Balb c and Swiss Webster). Sera have been assessed for specific binding to endogenous *Drosophila* PIE protein by immunostaining of eye imaginal discs from wild type. It is anticipated that the *pie* gene would be expressed in the developing eye, based on the mutant phenotype. As a control the same immunostaining have been performed with eye imaginal discs from homozygous $pie^{EB3}$ larvae. The $pie^{EB3}$ mutation is predicted to truncate PIE protein after Pro392, and so should not

be detected by antibodies raised against GST-PIE(F531-S582). So far no PIE-specific sera have been obtained, however.

Possible explanations for the failure yet to obtain PIE specific antisera include: the GST-PIE(F531-S582) protein may not be antigenic in mice; the PIE protein may be uniformly expressed at a low level, so that no difference from control staining has been discerned; the sera may contain other antibodies reacting with *Drosophila* tissues that masks underlying PIE-specific staining. To distinguish these possibilities we are now analyzing sera using western blots of purified bacterial GST-PIE(F531-S582) proteins. PIE-specific reactivity can be detected by western blotting, then we will attempt to define the subcellular location of the PIE protein by cell fractionation and western blotting experiments.

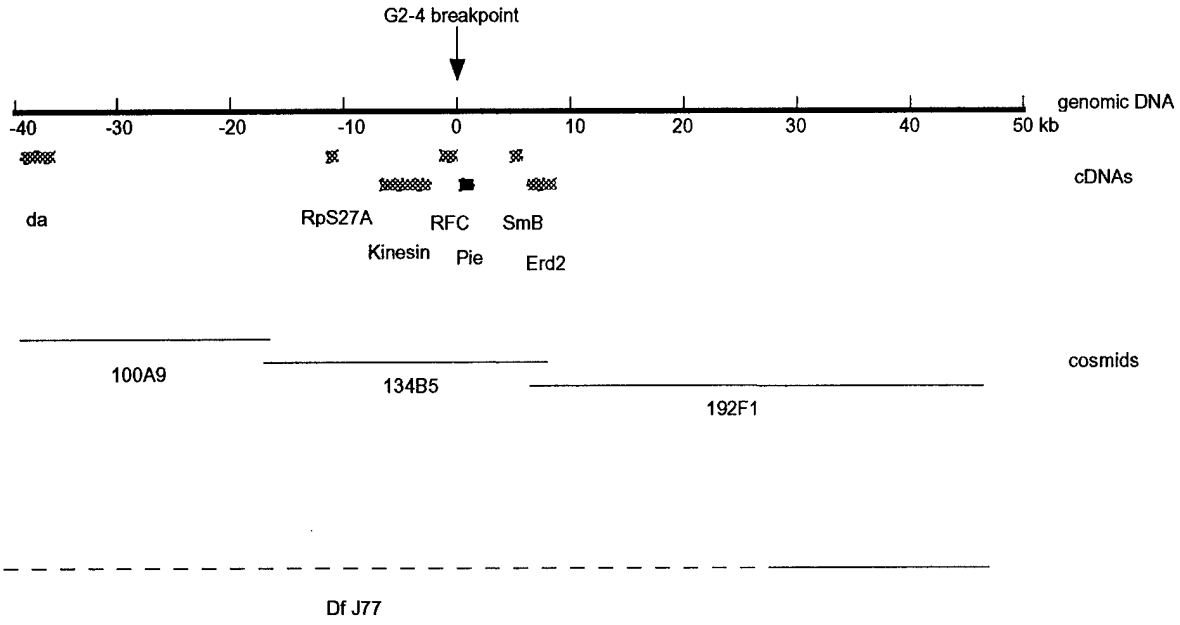
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**Figure 1**

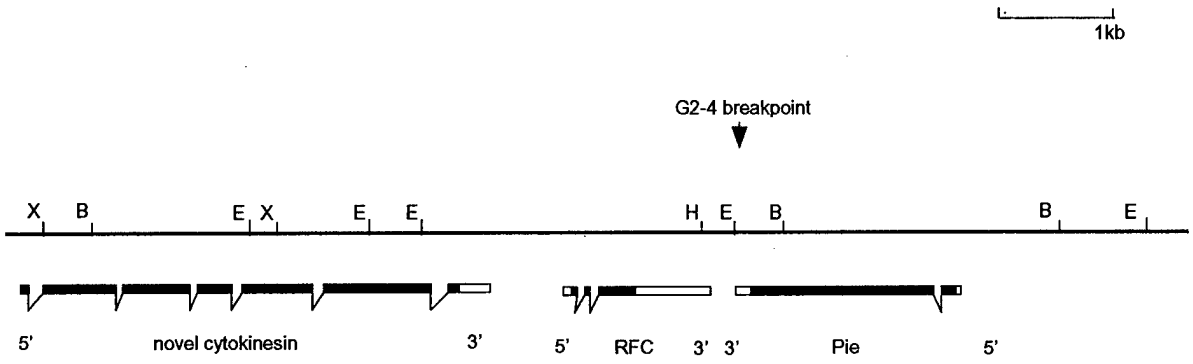
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Figure 2

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1    MEDNKELQCL ICKYSDTDDL VFGEWMIVRN LQVHYFCLLL
41   STHLPQRGGD SSGILGFLLR DIREEAAAAE KRKCWYCNKI
81   GASLQCDRCR SLFHLKCGLE NRAVFEFCGQ YKSYCYKCRP
121  MDDYKRQLQS KPPRNATCPI CFSSIYKVEL HCVVYGDCCR
161  LGFAHKKCMR QYALTSGYYL RCIWCRSERF RDSIRLQSVF
201  VPDRDATWEK QRNAYRELHE RNLKCDQPNC LCPSGRTYNR
241  LSWVILCCSS CAATSAHLKC LVGALRLPKK RERTDFKCSM
281  CLDVERRIAE GPARTTEETN ADGDNQVDGS FYVQKLGPDA
321  ATRSLTQTPV FSEEDESERS SNITVIFSQP KSNATSERLS
361  LSPPQEEMIV EIPDSPEASP KTSIDENHSP QPIARRDISD
401  SPQPIAASEI PDSPQPTAAS EIPDLPQTTA INVNPELTQQ
441  TALNTIPHSP QPEASFPTQL VSQTFDSPQP QEQAVAEAPN
481  SPSLPKEDPN TLLVLKSGFQ CPGEPFFYLV IYEFEHGTCM
521  GECIGTCVLR FKEDDPRIQD TSQAALERVK ITPDDVWCRS
561  EDRGIFEHIE KFHEWYRSEG FS
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Figure 3 Predicted PIE protein

Consensus amino acids for molybdopterin binding highlighted

Key Research Accomplishments

Identified the *pie* gene as a gene required for cell survival during development of *Drosophila melanogaster*.

Showed that *pie* and $l(2)31E_k$ are one and the same locus.

Cytologically and molecularly characterized the chromosome inversion $In(2L)32E_k{}^{G2\text{-}4}$:31E,32F.

Identified and characterized the *pie* gene molecularly, deducing its predicted product to be a novel 582 amino-acid protein.

Developed evidence against the notion that differential *pie* expression distinguished episodes of death and survival, favoring the view that *pie* is necessary but not sufficient for cell survival.

Reportable Outcomes

Sequence of the *pineapple eye* gene from *Drosophila melanogaster* , Genbank Accession Number AF247501.

Sequence of a kinesin-like gene from *Drosophila melanogaster* , Genbank Accession Number AF247500.

Sequence of Replication Factor C subunit 3 from *Drosophila melanogaster* , Genbank Accession Number AF247499.

Poster Presentation, Department of Defence "Era of Hope" Breast Cancer Research Meeting, Atlanta GA June 2000.

Conclusions

We have characterized the novel *pie* gene from *Drosophila,* and determined the basis for reduced cell number and developmental delay in *pie* mutants. We find that *pie* gene function is necessary for survival. The gene has been cloned and predicted to encode a novel 582 amino-acid protein with an unusual domain structure. There is a possibility based on sequence data that *pie* may encode a new molybdoprotein enzyme. These findings will be submitted for publication after further experiments to determine the subcellular location of the PIE protein. This further work is needed to complete evaluation of the role of the PIE protein in cell survival.

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Appendix 1

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Meeting Abstract

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Baker, Dr. Nicholas E.
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