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Genetics

pokkuri, a *Drosophila* gene encoding an E-26-specific (Ets) domain protein, prevents overproduction of the R7 photoreceptor

(neuronal fate determination/compound eye/transcription factor)

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ABSTRACT **Studies on sevenless and bride of sevenless genes have revealed that the R8 cell plays a key role in the fate of the R7 photoreceptor cell, presenting on its surface an inductive cue to which R7 responds. *sev*-independent induction of R7 cells has been reported in the seven-up mutation, which appears to transform R1 and R6 cells to R7 cells. We have induced recessive mutations in a gene pokkuri (*pok*; pokkuri is a Japanese word that means "dropping dead") that lead to overproduction of R7 cells with rather minor effects on outer photoreceptors and R8 cells. Pok protein may function as a transcription factor, as the predicted amino acid sequence contains a region similar to the consensus established for the E-26-specific (Ets) domain.**

Induction of R7 cells has been the most extensively analyzed event in the "stereotyped," sequential fate determination of *Drosophila* photoreceptors. The bride of sevenless (*boss*) gene product generated in R8 cells may act as a membrane-bound ligand for Sev, a receptor tyrosine kinase localized in the R7 precursor membrane that contacts R8 cells (1, 2). In fact, Krämer *et al.* (3) have recently demonstrated that Boss-bound Sev protein can be internalized by cultured S2 cells stably transfected with a plasmid containing sev cDNA as well as the R7 precursors *in vivo*. From these and other experiments, the *boss–sev*-mediated pathway has been suggested to be necessary and sufficient for R7 induction (but see ref. 4). We show that R7 cells can be produced in the absence of sev^+ by disrupting the pokkuri (*pok*) gene (22C). Mosaic analysis shows that no photoreceptor cell absolutely requires pok^+ for proper ommatidial development, although most (≈97%) R1 and R6 cells in normally developed mosaic eyes are genotypically wild type. The *pok* gene† encodes an E-26-specific (Ets) domain protein (5), implying a role as a transcription factor that provides a developmental cue.

MATERIALS AND METHODS

Mutagenesis, Mutant Screening, and Phenotype Analysis. The jump-start method (6) was used for mutagenesis with the *BmΔ–w* transposon (7) as a mutator. All flies used had a white$^-$ (w^-) background, whereas the *BmΔ–w* fly carried a copy of w^+, allowing us to recover chromosomes with *BmΔ–w* insertions by selecting individuals with nonwhite eye color. The resulting 1000 stocks with *P*-element insertions were subjected to screens for longevity at the adult stage. This strategy is based on the fact that some developmental mutants with defects in the adult nervous system [e.g., drop dead (8), *abl* (9), and seven in absentia (10)] are accompanied by reduced lifetime after eclosion. pok^1 and pok^3 were isolated as strains with extremely short life spans. Fifty percent mortality was attained at 2 days after eclosion in both strains. The mechanism for premature adult death is unknown. The *pok* mutations also reduce the viability of larvae and pupae: 25% of pok^1 homozygotes died at the larval stage, and 37% died in pupae. By introducing the Δ2.3 chromosome to the pok^1 line, the mutator element was remobilized, resulting in several lines with white eyes. pok^2 and pok^{sr15} were representative of these lines: pok^2 retained mutant phenotypes, whereas pok^{sr15} had a reasonably long lifetime (50% of tested flies survived for 1 mo) and normal eye morphology (Fig. 1*l*). Fixation and sectioning of heads from newly emerged adults were as described (1). Eye structures of newly emerged and aged *pok* mutant flies did not differ. Immunostaining and detection of *Rh3–lacZ* expression were done exactly as described (10, 12).

Mosaic Analysis. Heterozygotes for mosaic analysis were generated by crossing *w*; *P*[(w^+, ry^+)D]1 (inserted into the 25C region) to *w*;pok^2/SM1. The recombination distance between the *pok* gene and the white marker was estimated to be 10 map units. X-irradiation (1000–1200 rad; 1 rad = 0.01 Gy) of the progeny was done between 0 and 48 hr of development. The frequency of generating mosaic eyes was ≈1/500. Serial 1.5-μm sections were obtained along the long axis of the eye. Individual ommatidia were identified and followed from distal (≈10 μm in depth from surface) to proximal (≈90 μm from surface) in ≈15-μm steps to avoid error in scoring the presence of pigment.

Molecular Analysis. The plasmid-rescue method (13) was used to recover the pok^1 genomic DNA flanking the *P*-insertion point. The *pok* gene was localized at 22C on the chromosome 2L by *in situ* hybridization, with the rescued DNA as probe. Methods for extraction and analysis of RNA and genomic DNA were as described (13). By using an eye-disc cDNA library, 1×10^6 phages were screened, and four pok cDNAs were isolated. The pok cDNAs were subcloned into pUC19, and the nucleotide sequence was determined by the dideoxynucleotide chain-termination method with Sequenase (United States Biochemical).

RESULTS

Adult Eye Morphology of pok Mutants. The eyes of adult flies homozygous for pok^1 or pok^3 had a rough appearance, but no visible defects appeared in other external structures. The mutant ommatidia typically contained an excess number of rhabdomeres (Fig. 1*b*). In fewer instances, ommatidia with less than eight rhabdomeres were found. In addition, rhabdomeres of aberrant shape were often found (Fig. 1 *b* and *d*; wedges). In contrast, pok^{sr15} strain showed normal eye morphology with eight rhabdomeres per ommatidium (Fig. 1*l*).

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Abbreviation: Ets, E-26-specific domain.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. D10228).

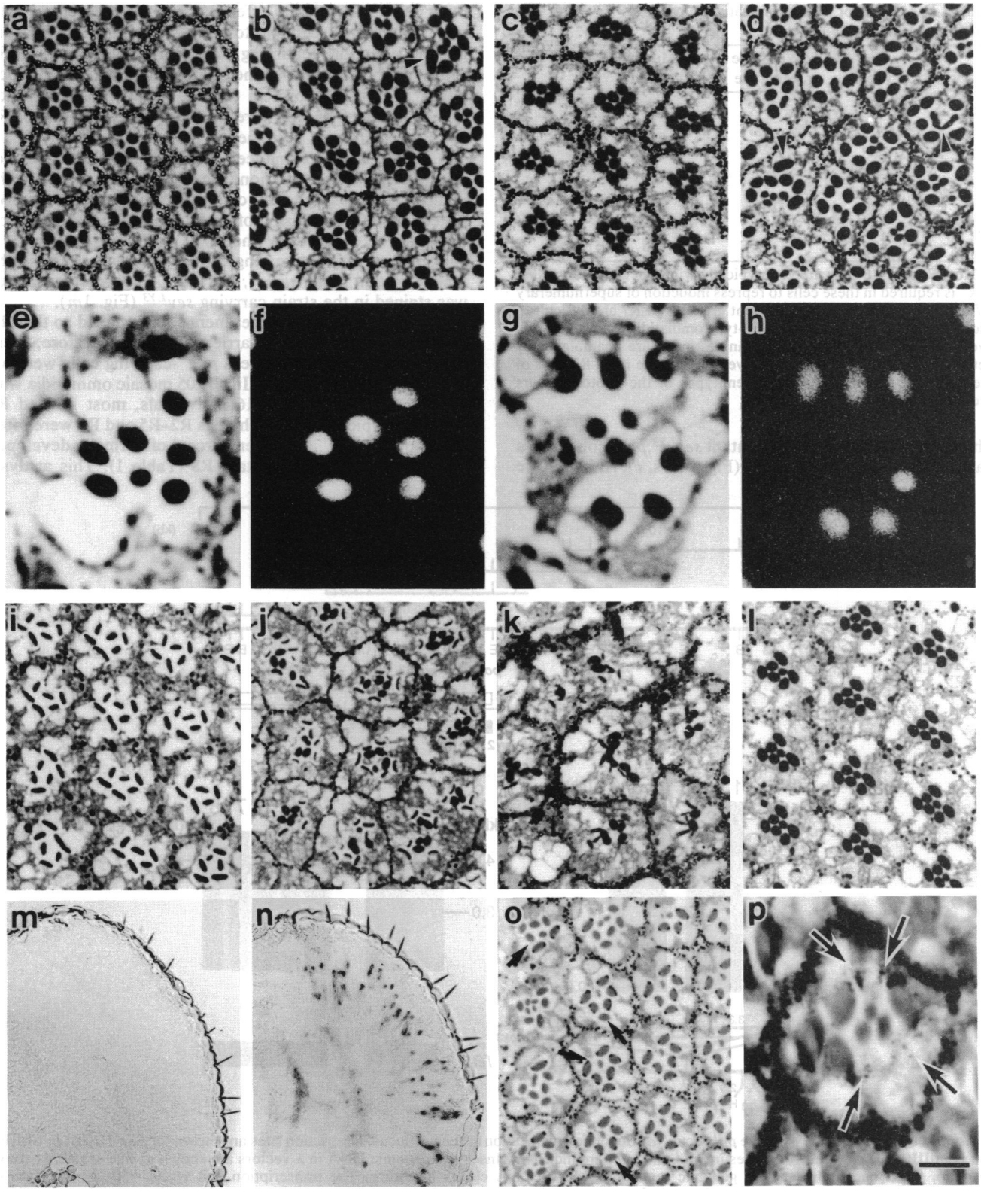

FIG. 1. Phenotype analysis. Tangential sections through distal portion of retina. Genotypes of the flies are: pok^1/pok^+ (*a*), pok^1/pok^1 (*b*), sev^{LY3}/sev^{LY3} (*c*), and sev^{LY3}/sev^{LY3}; pok^1/pok^1 (*d*). Rhabdomeres with aberrant morphology are found in mutant ommatidia (wedges). (*e*) A wild-type (Canton-S) eye stained with toluidine blue. (*f*) The same section as *e* stained with an antibody against Rh1 protein. (*g* and *h*) A pok^1 ommatidium stained with toluidine blue (*g*) or the antibody (*h*). (*i–l*) Distal sections through the ommatidia of sev^{LY3}; ora^{JK84} (*i*), pok^1; ora^{JK84} (*j*), sev^{LY3}; pok^1; ora^{JK84} (*k*), and pok^{sr15} (*l*). (*m* and *n*) *Rh3–lacZ* expression in retina. Frozen head sections stained for β-galactosidase activity of sev^{LY3} (*m*) and sev^{LY3}; pok^1 (*n*). The marker line used was *P[Rh3.2600lacZ]* (11). (*o*) A mosaic eye, illustrating the border between wild-type and mutant tissues. Phenotypically normal mosaic ommatidia (arrows) are seen with mutant R2–R5 and/or R7 and wild-type R1 and R6 (R8 is not observable in this section). (*p*) A phenotypically mutant mosaic ommatidium with multiple wild-type R7 cells. Pigment granules of three R7 cells are indicated with arrows. (Bar = 8.3 μm for *a–d* and *i–l*; 3.3 μm for *e–h*; 50 μm for *m* and *n*; 10 μm for *o*; and 5.3 μm for *p*.)

Table 1. Analysis of mosaic ommatidia having normal morphology

R cell	Wild-type genotype	Mutant genotype
R1	102	3
R2	64	41
R3	39	66
R4	50	55
R5	71	34
R6	103	2
R7	69	36
R8	85	20

Most R1 and R6 cells are genotypically wild type, suggesting that pok^+ is required in these cells to repress induction of supernumerary R7 cells. In some cases, the pigment granules of R7 are difficult to score. We examined at least 20 wild-type ommatidia for each mosaic eye to see whether the pigment granules in R7 are unambiguously detected. Only when the granules were detectable in all R7 cells of the wild-type ommatidium were genotypes of the photoreceptors scored.

The ommatidia of the double mutant sev^{LY3};pok^1 contained a variable number of R7-like cells (Fig. 1 *d* vs. *c*). Although the outer cells were clearly affected (Fig. 1), the central cells were far more susceptible to the *pok* mutations. There are good reasons for identifying most pok^1-induced cells as R7 cells. (*i*) They are not stained with a monoclonal antibody raised against Rh1 protein (Fig. 1 *g* and *h*), with which rhabdomeres of R1–R6 were labeled, whereas those of R7 and R8 cells remain unstained in the wild-type eye (Fig. 1 *e* and *f*). (*ii*) The *pok*-induced small rhabdomeres are not affected by the *ora* mutation (Fig. 1 *i–k*), indicating that the induced cells belong to the central photoreceptor class. We tested whether expression of an R7 marker gene, *Rh3–lacZ* (11), can be induced by the pok^1 mutation in the sev^{LY3} background. *lacZ*-expressing cells were clearly seen in the double mutant strain sev^{LY3}; pok^1 (Fig. 1*n*), whereas no cell was stained in the strain carrying sev^{LY3} (Fig. 1*m*).

Mosaic Analysis. The experiment was designed so that the cells carrying pok^+ also carried w^+ and, therefore, were labeled with pigment, whereas pok^--carrying cells were w^- and unpigmented (Fig. 1*o*). In all 105 mosaic ommatidia with normal morphology from 16 individuals, most R1 and R6 were genotypically pok^+, whereas R2–R5 and R7 were often genotypically pok^-. Nineteen percent of normally developed mosaic ommatidia had mutant R8 (Table 1). This analysis

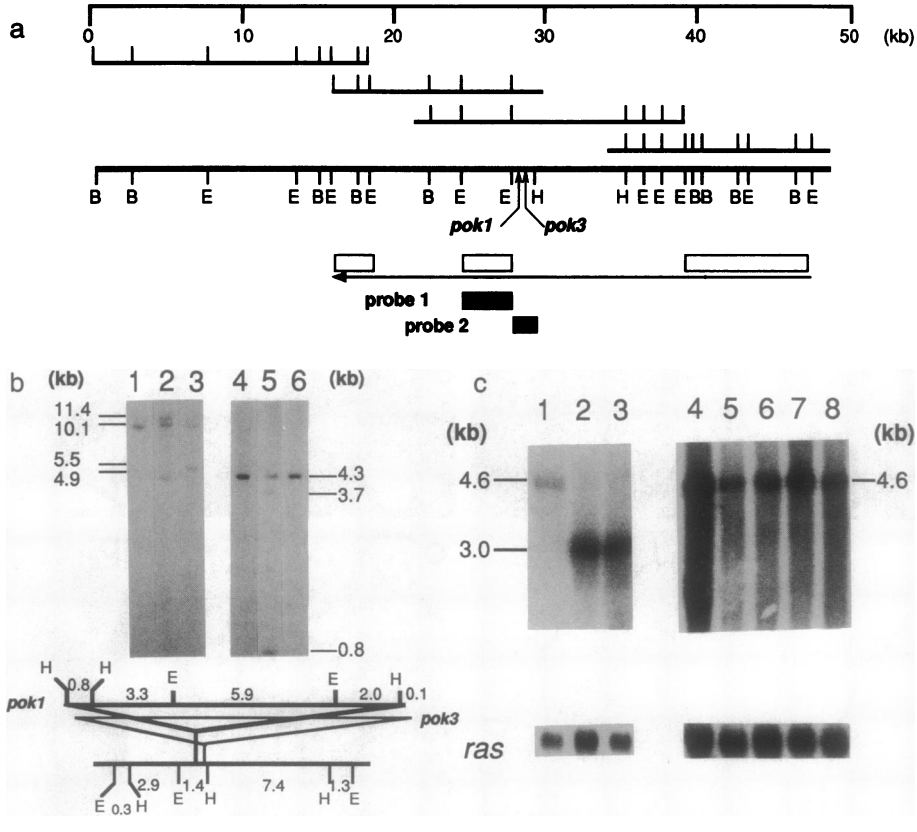

FIG. 2. Molecular analysis of the *pok* locus. (*a*) Genomic organization at the *pok* locus. Restriction sites are shown for *Bam*HI(B), *Eco*RI(E), and *Hin*dIII(H). The *P*-insertion sites in pok^1 and pok^3 are indicated. Inserts of genomic DNA in λ vectors are shown as line segments above the map. Open bars represent the genomic regions to which cDNA clones hybridize. The transcription unit spans ≈30 kb; direction of transcription is indicated by an arrow. Genomic fragments used as probes for RNA and Southern blot analyses and for screening of a cDNA library are also shown (solid bar). (*b*) Mapping the *P*-insertion points in pok^1 and pok^3. DNA from wild type (lanes 1 and 4), pok^1/SM1 (lanes 2 and 5), and pok^3/SM1 (lanes 3 and 6) was digested with *Eco*RI (lanes 1–3) or *Hin*dIII (lanes 4–6) and hybridized with probe 2. The balancer chromosome in pok^1 and pok^3 heterozygotes produced bands identical to those in the wild type (10.1 kb for lanes 2 and 3; 4.3 kb for lanes 5 and 6). An *Eco*RI site at the 5′ end of the mini-white gene in the transposon (see diagram at bottom) yielded unique bands at 4.9 kb for pok^1 strain (lane 2) and 5.5 kb for pok^3 strain (lane 3), reflecting the distance between two insertion points. A *Hin*dIII site in the pUC sequence created additional fragments in *Hin*dIII digests from pok^1 (lane 5) and pok^3 (lane 6). Molecular structures of pok^1 and pok^3 chromosomes are diagrammed below. (*c*) RNA blot analysis of the *pok* transcript. (*Left*) Transcripts in wild type (lane 1), pok^1 (lane 2), and pok^3 (lane 3) adult flies. (*Right*) Transcription of the *pok* gene throughout development: 0- to 12-hr embryo (lane 4), 12- to 24-hr embryo (lane 5), 3rd instar larva (lane 6), pupa (lane 7), and adult (lane 8). The same blots probed with the *ras* DNA are shown below. Ten micrograms of poly(A)$^+$ RNA was loaded in each lane and hybridized with probe 1. Both pok^1 and pok^3 strains have at least 5-fold more abundant 3.0-kb transcript than the 4.6-kb transcript detected in wild type, suggesting that the normal transcriptional regulation of the gene is perturbed.

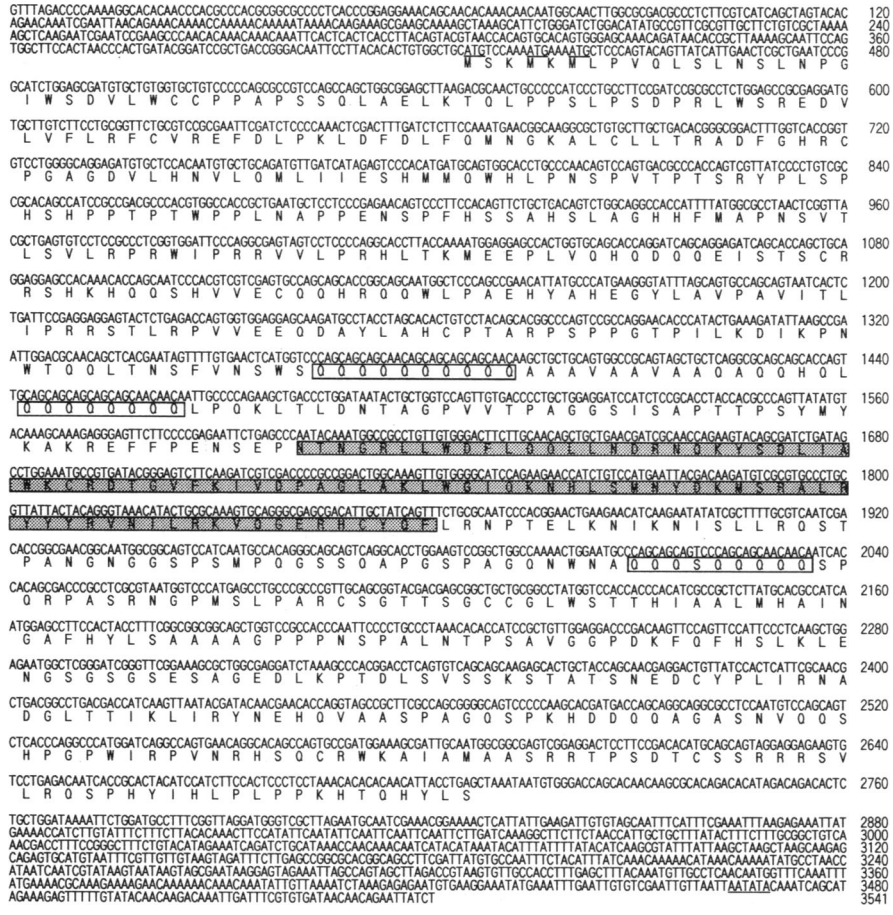

FIG. 3. Structure of pok cDNA and predicted amino acid sequence. Underlined sequences include potential translation initiation codons and the putative polyadenylylation signal. The predicted amino acid sequence is indicated with one-letter code. Three *opa* repeats are boxed, and the Ets domain is shaded. Four clones were used: clone 1 covering nucleic acid bases −558–1590; clone 2 covering bases −152–3311; clone 3 covering bases 1–3541; and clone 4 covering bases 174–1590.

clearly establishes that normal development of ommatidia is independent of pok^+ expression in R2–R5 and R8. Instead, pok^+ gene function expressed in the R1 and R6 cells appears necessary for development of other photoreceptors. In mosaic ommatidia that are phenotypically mutant, more than one R7 cell can be genotypically wild type (Fig. 1*p*). This observation makes it less likely that the Pok protein wild-type function is required in all cells that have the potential to become R7, including cone and R7 precursors. However, both in pok^- mosaics and mutant homozygotes some ommatidia developed normally.

Isolation of pok Genomic DNA and Identification of the pok Transcript. Approximately 50 kilobases (kb) of chromosomal DNA encompassing the *P*-insertion sites was cloned, and the restriction map was determined (Fig. 2 *a* and *b*). In the two mutant strains pok^1 and pok^3, *P*-element insertions were found for both to lie within 600 base pairs (bp) of one another in the 1.4-kb *Eco*RI–*Hin*dIII fragment (Fig. 2*b*). No other restriction fragment polymorphism was found between genomic DNA of Canton-S, pok^1, and pok^3. These results strongly suggest that this region of DNA harbors the *pok* gene. Among transcripts detected in this region, only one was affected by the *pok* mutations. The 4.6-kb transcript, detected in the wild type by the 3.2-kb *Eco*RI fragment adjacent to the *P*-insertion site (probe 1; Fig. 2*a*), was undetectable in pok^1 and pok^3 strains. Instead, an altered transcript of 3.0 kb was found in both strains (Fig. 2*c*). The *P* elements have been inserted into an intron of the *pok* gene in both cases (Fig. 2*a*). The truncated transcript might result from improper transcription termination or imprecise splicing of mRNA. We conclude that the 4.6-kb message is the *pok* transcript because it derives from the genomic region with *P*-element insertions that produce mutant phenotypes. The normal *pok* transcript is undetectable in two mutant lines. The *pok* transcript was present throughout the developmental stages tested (Fig. 2*c*).

Isolation of pok cDNA and Deduced Polypeptide Sequence. Four overlapping cDNA clones corresponding to the *pok* transcript were isolated and confirmed on RNA blotting to hybridize with the same transcript as detected by the genomic DNA probe (probe 1). The *pok* transcript appears to have an extremely long 5′ leader sequence (>1 kb) with several ATG-initiated open reading frames interrupted by the stop codons existing immediately after the start sites. The longest potential open reading frame can encode a putative protein 761 amino acids long with a calculated molecular mass of 84.6 kDa (Fig. 3). The molecular mass of the Pok protein produced in *Escherichia coli* is 88 kDa, and its N-terminal sequence (down to 15 residues) is identical to that deduced from the cDNA sequence (H.T., unpublished data). The polypeptide has three *opa* repeats. The most striking feature of the amino acid sequence is an 87-amino acid Ets domain (5) (Fig. 4*a*). The Ets domain is a domain characterized by three tryptophan residues ≈18 amino acids apart and with basic amino acids concentrated in the carboxyl half of the domain. The Pok Ets domain most closely resembles that of the human *ERG* gene product with amino acid identity of 46.0% (Fig. 4*a*). When conserved substitutions of amino acids are taken into account, similarity of the two sequences exceeds 64.4%. Similarity of Pok protein and other members of the family

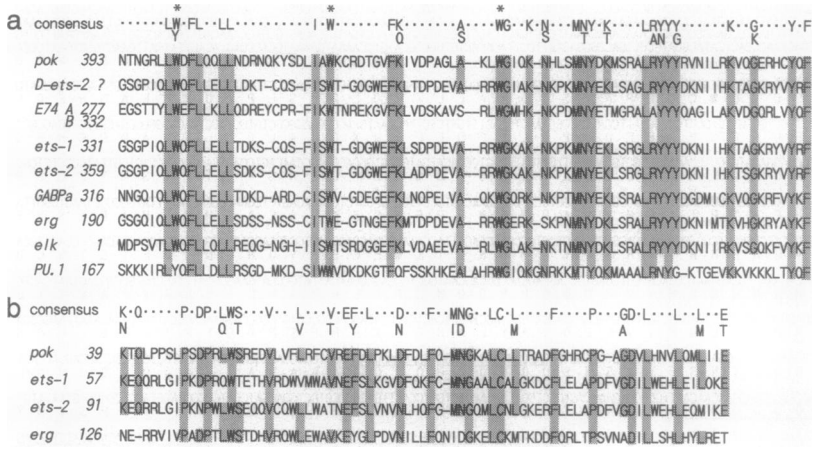

FIG. 4. Alignment of conserved amino acid sequences: *Drosophila* Ets-2, *Drosophila* E74, murine Ets-1, human ETS2, murine GA (guanine-adenine)-binding protein α subunit (GABPα), human ERG, human ELK, and murine purine-rich sequence (PU)-box binding protein 1, (PU.1). (*a*) Amino acid sequences within these Ets domains. Listed at top is the consensus sequence, which consists of the 18-amino acid identities and some positions with conservative amino acid substitutions. The conserved tryptophan residues (*) are indicated above the consensus. Listed at left of each sequence is the name of the gene and the number of amino acids away from the putative start codon. (*b*) Comparison of the N-terminal region of Pok with other Ets family proteins.

extends beyond the Ets domain: the N-terminal region of the Pok protein contains an ≈80-amino acid stretch resembling that of *c-ets-1*, *c-ets-2*, and *erg* (5) (Fig. 4*b*)-encoded proteins.

DISCUSSION

One of the most important findings from this study is that R7 cells can be induced without sev^+, provided the pok^+ gene function is eliminated. There is an intriguing possibility that a cell population has the potential to become R7 type, unless the *pok*-mediated inhibitory signal is received. The origin of additional R7 cells in *pok* mutants remains to be elucidated. Mosaic analysis suggests that the wild-type function of *pok* in R1 and R6 cells is important in blocking the induction of extra R7 cells. It has been reported that the absence of svp^+ function causes a transformation of, at most, four outer photoreceptors toward an R7 cell fate (4). Extra R7 cells are probably not produced in the *pok* eyes by a similar transformation because the mutant ommatidia contain, on average, six outer photoreceptors. However, we cannot exclude the possibility that some outer cells develop as R7 in the *pok* ommatidia. Loss of the proper outer cells could be obscured by a compensatory transformation of nonneural cells (e.g., "mystery cells": cell aggregates commonly contain one or two extra mystery cells, which are eliminated to form the precluster, see refs. 4 and 14) into outer photoreceptors. A preliminary experiment has revealed that extra monoclonal antibody 22C10-positive cells are already present at columns 5 and 6 posterior to the furrow in some clusters of mutant eye discs. Whether the additional neural cells take on the R7 fate or the R1–6 outer cell fate will be interesting to determine.

The *pok* gene encodes an Ets-domain protein. Although the function of the Ets-related proteins has not been firmly established, several lines of evidence suggest that they form a complex with other proteins to function as transcriptional regulators, and the Ets domain serves as the DNA-binding motif (15). If this is so for *pok*, then questions arise: which are the target genes and what are the interacting proteins? Isolation of dominant modifiers of the *pok* phenotype followed by molecular cloning of these genes may enable us to answer such questions and provide further insights into a general mechanism for orchestration of genes in controlling development.

Note Added in Proof. The *yan* gene, isolated independently by Z.-C. Lai and G. M. Rubin (personal communication), appears to be the same gene as *pok*.

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