

The *Drosophila Ras2* and *Rop* gene pair: a dual homology with a yeast *Ras*-like gene and a suppressor of its loss-of-function phenotype

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SUMMARY

The promoter of the *Drosophila melanogaster Ras2* gene is bidirectional, regulating an additional gene oriented in the opposite polarity. The two divergently transcribed genes are only 93 bases apart and deletion analysis proved that common *cis*-acting elements within this promoter region are required for the transcriptional activity of both genes. We cloned the gene paired with *Ras2* in the bidirectional promoter and isolated cDNAs corresponding to its mRNA. The *Ras* opposite (*Rop*) gene encodes for a $68 \times 10^3 M_r$ protein which shares sequence homology with the members of a novel *Saccharomyces cerevisiae* gene family, including the *SLY1*, *SEC1* and *VPS33* (*SLP1*) genes, all of which are involved in vesicle trafficking among yeast cellular compartments. A highly conserved motif in this family is also found in β -COP, a coat protein isolated from rat Golgi-bound non-clathrin vesicles. Thus, the *Rop* protein may be a component of one of the vesicle trafficking pathways in *Drosophila* cells. The *Rop* gene expression during embryogenesis is restricted to the central nervous

system (CNS) and the garland cells, a small group of nephrocytes that takes up waste materials from the haemolymph by endocytosis. *Ras2* is also expressed in the embryonic garland cells. In postembryonic stages, the two genes are co-expressed in the larval salivary glands and the central nervous system, and in the adult CNS and reproductive systems. Interestingly, the *S. cerevisiae SLY1-20* allele is a suppressor of the loss of the *YPT1* gene, a *ras*-like gene implicated in vesicle translocation, suggesting that the two genes may interact with one another. Since Sec1p and β -COP may also interact with small GTP-binding proteins of the *ras* superfamily, it is conceivable that the *Rop* and *Ras2* gene products are not just co-expressed in common tissues, but may also functionally interact with one another in these tissues.

Key words: *Drosophila*, *Ras* gene, bidirectional promoter, vesicle trafficking, garland cells, endocytosis

INTRODUCTION

Three *D. melanogaster Ras* genes, *Ras1*, *Ras2* and *Ras3/Rap1*, were cloned and characterized (reviewed in Lev, 1993). Analysis of their sequence homology to other *ras*-like genes indicated that they are related to different families in the *Ras* branch of the *ras* supergene family. *Ras1* belongs to the *Ras* family, which includes the three human transforming *ras* genes and the yeast *RAS* genes. *Ras2* belongs to another gene family, which contains the human non-transforming R-*ras* gene and the TC21 gene. *Ras3/Rap1* is most similar to the *Rap* family which contains the *rap1/Ki-rev* gene and other *rap* and *smg* genes (Chardin, 1991; Valencia et al., 1991).

Studies on the developmental regulation of the *Drosophila Ras* genes have shown that they are expressed constitutively during all developmental stages and are moderately abundant in poly(A) RNA (Lev et al., 1985; Mozer et al., 1985). However, additional studies bearing on their

expression in *Drosophila* tissues have indicated that they are spatially regulated. Thus, all three genes are expressed in a relatively small number of tissues, including undifferentiated proliferating cells and terminally differentiated tissues (Segal and Shilo, 1986).

The *Ras1* gene participates in the signal transduction pathway determining the fate of the R7 photoreceptor in the *Drosophila* compound eye (Simon et al., 1991; Fortini et al., 1992; Gaul et al., 1992). *Ras3/Rap1* may act as an inhibitor of this process (Hariharan et al., 1991). The *Ras2* gene does not participate in this pathway (Fortini et al., 1992), but there are indications for its involvement in other proliferative pathways. Transgenic *Drosophila* strains containing the activated form of the *Ras2* protein have low fertility and show developmental disturbances in their bristles, wings and eyes (Bishop and Corces, 1988). However, as yet no specific role has been assigned to this gene.

During the isolation and characterization of the *Drosophila Ras2* promoter region, we found that, in addi-

tion to *Ras2*, another gene is transcribed in this region, but in the opposite polarity relative to the *Ras2* gene (Cohen et al., 1988). *Ras2* and the divergently transcribed gene, temporarily termed *CS1* and now termed *Ras opposite (Rop)*, are merely 93 nucleotides apart. Deletion analysis has proved that certain *cis*-acting elements within this promoter are required for full transcriptional activity of both genes simultaneously (Lev et al., 1989). Thus, *Ras2* and *Rop* are regulated by a genuine bidirectional promoter.

Here, we report on the cloning and sequencing of the *Rop* gene and the pattern of its expression during *Drosophila* development. The *Rop* protein shows significant homology with the members of a novel *S. cerevisiae* gene family involved in vesicle trafficking among yeast cellular membranes (Aalto et al., 1992). The *Rop* and *Ras2* gene products are co-expressed in several embryonic, larval and adult tissues, including the garland cells, a group of nephrocytes implicated in the removal of toxic materials from the haemolymph by endocytosis (Aggarwal and King, 1967; Rizki, 1978).

MATERIALS AND METHODS

Drosophila strains

The OregonR wild-type strain was obtained from the CalTech *Drosophila* stock center. The *white* strain utilized for generating the transgenic lines was obtained from the collection of E. Liphshitz.

Isolation of *Rop* genomic and cDNA clones

The *Ras2/Rop* genomic region was isolated from a recombinant phage provided by B. Shilo. *Rop* cDNAs were isolated from a *D. melanogaster* embryonic cDNA library constructed by L. Kauvar and T. Kornberg, using DNA probes from genomic regions upstream of the *Ras2* transcription start site. *Rop*-positive clones were detected at a frequency of 1 in 15,000. Each part of the sequence was determined at least twice, in both directions.

Detection of the *Rop* protein

Full-length *Rop* cDNA was cloned in the vector pHSREM1 (Knipple and Marsella-Herrick, 1988) under regulation of the *Drosophila hsp70* promoter. Schneider S2 cells stably transformed with this construct were obtained by co-transfection with the pPC4 vector (Jokerst et al., 1989) followed by selection for α -amanitin resistance, as described (Rio and Rubin, 1985).

To prepare anti-*Rop* antibodies the *Rop* cDNA fragment coding for amino acids 51 to 492 was cloned in frame with the N terminus of the *E. coli trpE* protein in the expression vector pATH11 (a gift of A. Tzagoloff). The fused *trpE-Rop* protein produced in MH1 bacteria was isolated and used to raise rabbit anti-*Rop* antibodies.

Total proteins from S2 cells or *Drosophila* adult flies were solubilized in lysis buffer, electrophoresed in 9% polyacrylamide-SDS gel, blotted, reacted with polyclonal anti-*Rop* antibodies and the *Rop* protein was detected by autoradiography or by the ECL system (Amersham). To obtain heat-induced *Rop* protein, the S2 cells were heat-pulsed for 15 minutes at 37°C and, after a recovery period of 90 minutes, total proteins were extracted.

In situ hybridization

DNA labeled with DIG-dUTP (Boehringer) was used as a probe for in situ hybridization essentially according to the published protocol (Tautz and Pfeifle, 1989). The *Ras2* probe was a 760 bp

EcoRI fragment from a *Ras2* cDNA clone, which contains the entire *Ras2* coding region. The *Rop* probe was a 1.65 kb *EcoRI* fragment from the coding region of a *Rop* cDNA clone. This fragment detects the two *Rop* transcripts.

Construction of *Ras2-lacZ* and *Rop-lacZ* transcription fusions

The *Ras2* promoter region was a 1030 bp *KpnI/DraI* fragment from pTZ18-1.9 (Cohen et al., 1988) and the *Rop* promoter region was a 1095 bp *KpnI/SpyI* fragment from SK-*Rop*-CAT(NcoI) (S. Ezer and Z. L., unpublished results). They were inserted into the vector pW-ATG-lacZ1 (Kuhn et al., 1988), between the *KpnI* and *Xba* sites. pW-ATG-lacZ1 was derived from the pW8 transducing vector (Klemenz et al., 1987). The *Ras2* promoter fragment in *Ras2-lacZ* spanned 852 bases upstream of the *Ras2* transcription start site and 78 bases downstream of this site. The *Rop* promoter fragment in *Rop-lacZ* consisted of 1047 bases upstream of the *Rop* transcription start site and 48 bases downstream of this site. Transgenic flies were obtained by injecting these constructs into *white* embryos as described (Spradling and Rubin, 1982; Spradling, 1986). The transformed lines were made homozygous and the chromosomes containing the transposons were determined by standard methods (Ashburner, 1989a). Additional lines were obtained by the jumping of transposons from the X chromosome to an autosome as described (Cooley et al., 1988).

Detection of β -galactosidase activity

Histochemical staining for β -galactosidase activity was carried out essentially as described (Ashburner, 1989b). Staged embryos were collected and washed with water. After dechorionation in 50% bleaching solution, they were extensively washed with water and fixed for 15-20 minutes in fixing solution (7% formaldehyde in 100 mM Pipes, pH 6.9, 2 mM EGTA, 1 mM MgSO₄) mixed with 2 volumes of n-heptane. They were washed three times in washing solution (PBS [50 mM Na/Na₂PO₄, pH 7.4; 140 mM NaCl] containing 0.3% Triton X-100) and stained for 4 to 18 hours in staining solution (Na/Na₂PO₄, pH 7.2; 150 mM NaCl; 1 mM MgCl₂; 3.1 mM potassium ferricyanide; 3.1 mM potassium ferrocyanide; 0.3% Triton X-100) containing 0.2% X-gal, at 37°C. After three washes in washing solution, the embryos were incubated in PBS containing 70% glycerol and mounted for observation. Dissected larval salivary glands, brains, imaginal discs and adult testes were fixed for 15-20 minutes in 100 mM sodium cacodylate, pH 7.3, containing 0.75% glutaraldehyde. The organs were washed in PBS and stained as described above. Dissected ovaries were incubated for 10 minutes in devitellinization solution (16 mM K/K₂PO₄, pH 6.8; 75 mM KCl; 25 mM NaCl; 3.5 mM MgCl₂; 6% formaldehyde) mixed with six volumes of n-heptane. The ovaries were fixed and stained as described above for the embryos preparation.

RESULTS

The *Rop* gene and its product

The *Drosophila Rop* gene was initially identified as a distinct transcription unit located upstream of the *Ras2* gene and divergently transcribed in opposite polarity to the *Ras2* gene (Cohen et al., 1988, Fig. 1A). The transcription start sites of the *Rop* and *Ras2* transcription units were determined to be only 93 bases apart (Cohen et al., 1988). Furthermore, data obtained from sequencing *Ras2* cDNAs (Bishop and Corces, 1988; N. H., unpublished results) and *Rop* cDNAs (see below) revealed that they start, as pre-

dicted, from the start site determinations described above (Fig. 1B). Under stringent hybridization conditions *Rop* appeared as a single-copy gene (A. S., unpublished results),

coding for two transcripts, 2.5 and 2.9 kb long (Cohen et al., 1988). These transcripts were initially assigned by others to *Ras2* (Mozer et al., 1985; Brock, 1987).

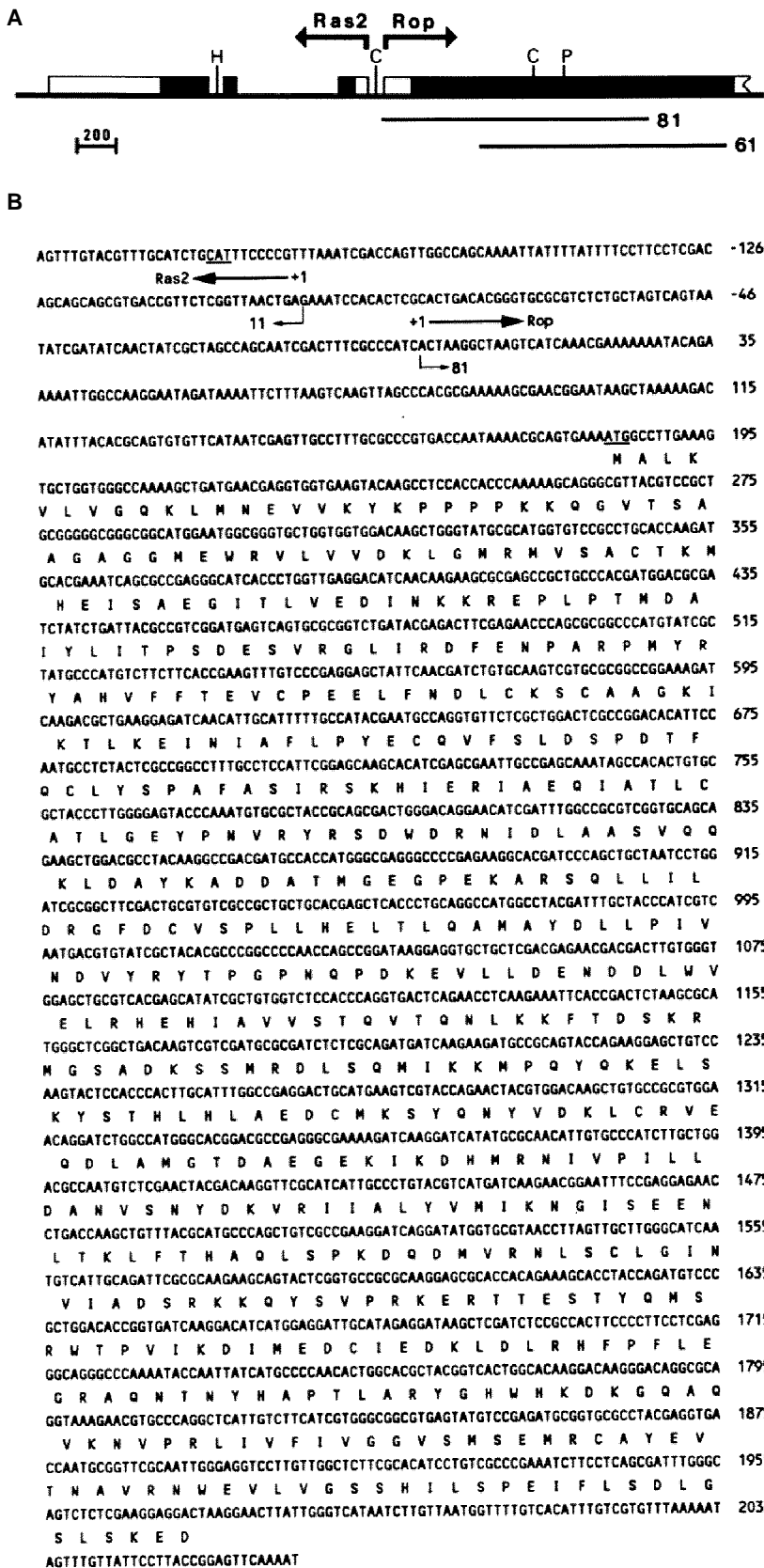


Fig. 1. The *Drosophila Rop* nucleotide and deduced amino acid sequence. (A) The organization of the *Ras2/Rop* gene pair region. The arrows denote the transcription start site of each gene (Cohen et al., 1988). Thick line, intron; boxed line, exon, untranslated; filled, translated. The 3'-end of the *Rop* transcription unit has not been determined; 81, 61, the *Rop81* and *Rop61* cDNAs used for sequencing. (B) *Rop* genomic and cDNA nucleotide sequence, and the deduced amino acid sequence of the *Rop* protein. Thin arrows above the sequence, transcription start sites, determined by external primer extension and RNase protection (Cohen et al., 1988). Underlines above the sequence, transcription start sites, determined by external primer extension and RNase protection (Cohen et al., 1988). The sequence of the promoter region contains a few corrections to the sequence published earlier (Cohen et al., 1988).

To learn about the identity of *Rop*, two overlapping *Rop* cDNA clones and the *Rop* genomic DNA region were isolated and sequenced in parallel (Fig. 1). The *Rop* translation start site is at position 183 and the ATG start codon is embedded in the sequence GAAAATG, which conforms in 6 out of 7 bases to the *Drosophila* translation start consensus sequence (Cavener, 1987). The open reading frame is 597 amino acids long, encoding for a protein with a predicted relative molecular mass of 67,800. The cDNA nucleotide sequence appears to be colinear with the genomic sequence along the 1950 bases sequenced, indicating that the translated portion of at least one *Rop* mRNA is transcribed from an intronless genomic DNA region. The *Rop* protein is slightly acidic (pI = 6.22) and is mostly hydrophilic, containing 26% charged amino acids. No transmembrane regions, conserved sites for nucleotide binding or enzymatic activity were found.

Homology of *Rop* with known proteins

A search of the SwissProt protein database revealed significant homology (FASTA score above 200) between the *Rop* protein and the products of a novel *S. cerevisiae* gene family including *SLY1*, *SEC1* and *VPS33(SLP1)*. All of them are genes associated with protein trafficking among yeast cellular membranes (Aalto et al., 1992). The *SLY1* gene product may be functional in endoplasmic reticulum (ER)-to-Golgi transport (Ossig et al., 1991). The *SEC1* gene product is also involved in protein secretion, but in a different step. It is one of the ten genes identified by the *SEC* (for secretion) mutations needed for the final stage of protein secretion in which the secretory vesicles fuse with the cell membrane to release the secretory proteins to the cell exterior (Novick et al., 1980; Aalto et al., 1991). The product of the third gene, *VPS33(SLP1)*, is involved in transport from the Golgi complex to the cell vacuole (Robinson et al., 1988; Wada et al., 1990).

Apparently *Rop* is the first member of this gene family to be identified in a multicellular organism. The highest homology in the family is among *Rop*, *SLY1* and *SEC1* (Fig. 2A). The *Rop* protein sequence appears to be even more similar to Sly1p and Sec1p than these two yeast proteins are to one another. There is 25% sequence identity (38% sequence similarity) between *Rop* and Sly1p, and 24% sequence identity (40% sequence similarity) between *Rop* and Sec1p. The homology of these proteins to VPS33p is slightly lower (not shown, but see Aalto et al., 1992). In some of the highly conserved regions the sequence identity reaches 50 to 70%. One of these regions is also present in -COP (H. D. Schmitt, C. Dascher and M. Egerton, personal communication) a $110 \times 10^3 M_r$ rat protein associated with non-clathrin-coated vesicles and the Golgi complex (Duden et al., 1991; Serafini et al., 1991).

Detection of the *Rop* protein

To identify the *Rop* gene product, anti-*Rop* antibodies were raised against a *trpE-Rop* fusion protein expressed in bacteria. The antibodies precipitated a single protein from metabolically labeled Schneider S2 tissue culture cells (Fig. 3A) with the predicted M_r of 68×10^3 . They also recognized

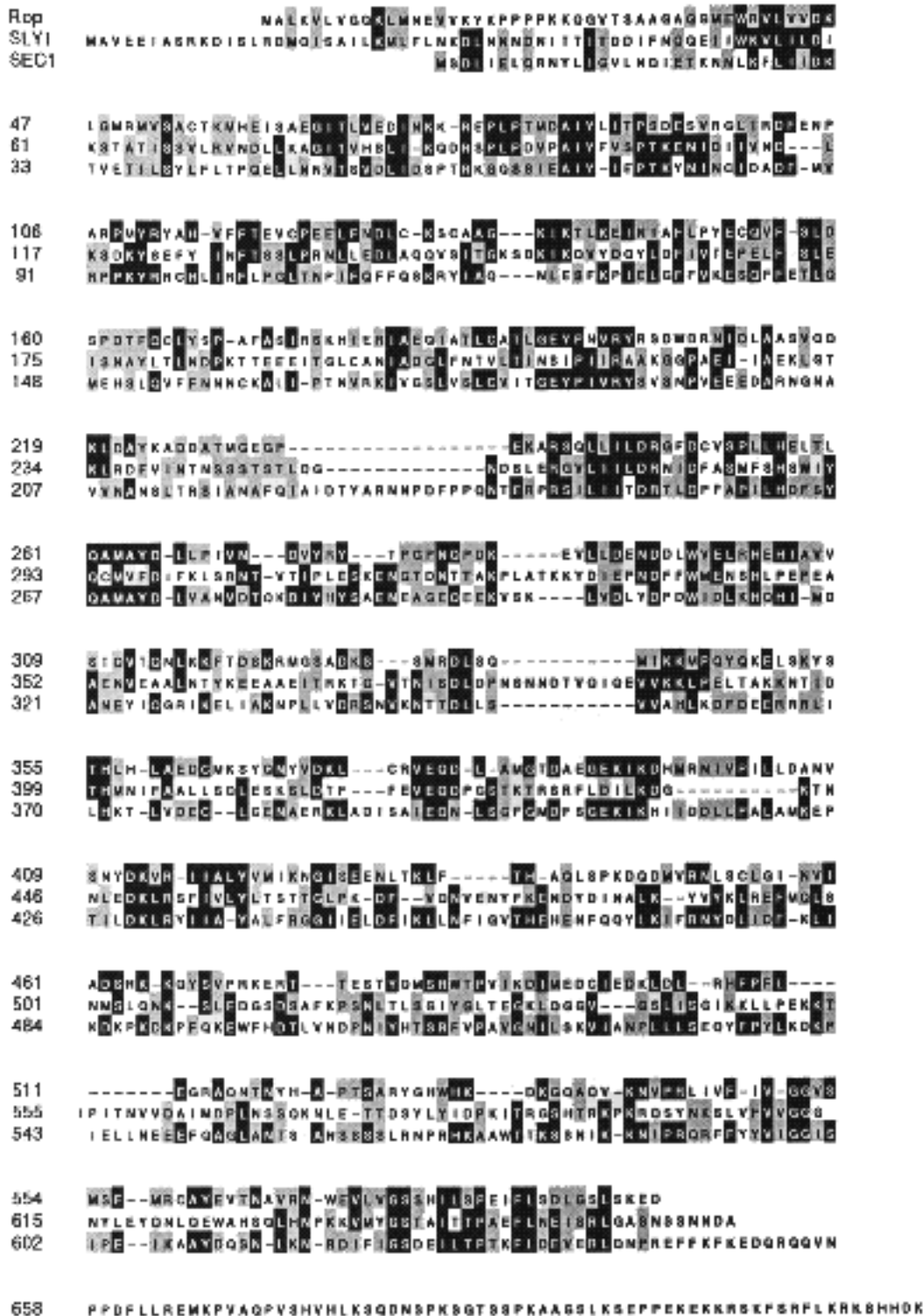
the expected $68 \times 10^3 M_r$ heat-inducible protein in a heat-pulsed Schneider S2 cell line stably transformed with a *Hsp70-Rop* transcription fusion (Fig. 3B). The *Rop* protein was also detected in adult flies, with higher levels in males than in females (Fig. 3C).

Localization of the *Ras2* and *Rop* transcripts during embryogenesis

To learn about the temporal and spatial distribution of the *Ras2* and *Rop* transcripts during embryogenesis, we utilized DNA fragments labeled with digoxigenin as probes for the in situ hybridization analysis. Hybrids formed between the probe and the endogenous transcripts were detected by anti-digoxigenin antibodies coupled with alkaline phosphatase (Tautz and Pfeifle, 1989). The *Ras2*-specific probe was capable of detecting the two transcripts coded by the *Ras2* gene (Lev et al., 1985). The *Rop*-specific probe could detect the two transcripts coded by the *Rop* gene (Cohen et al., 1988). To test the specificity of the hybridization reaction, similar experiments were carried out using a DNA probe prepared from the bacterial plasmid pBR322. In addition, to exclude the possibility of cross-reactivity with other *Ras*-like genes, we repeated these experiments with *Ras1* and *Ras3* probes. Distinct, different patterns were obtained for each of the *Ras* genes. Although the presence of *Ras2* and *Rop* transcripts during early embryogenesis has been established by RNA-blotting experiments (Lev et al., 1985; Mozer et al., 1985; Segal and Shilo, 1986), until stage 11 of embryogenesis (5.3 to 7.3 hours, end of germ band extension; Campos-Ortega and Hartenstein, 1985), the signal was homogeneous and apparently with an intensity similar to the control reaction (not shown).

The first indication of a specific signal was detected at stage 13 (9.3 to 10.3 hours, end of germ band retraction) where the garland cells were marked by the *Ras2* probe (Fig. 4A). The garland cells were clearly stained by the *Rop* probe as well (Fig. 4B). These nephrocytes are organized as a ring of cells around the oesophagus (Rizki, 1978; Fig. 5). They were implicated in the removal of toxic materials from the haemolymph by endocytosis (Aggarwal and King, 1967; Narita et al., 1989). The *Ras1* and *Ras3/Rap1* probes did not stain the garland cells. The *Rop* probe also marked cells in the central nervous system (Fig. 4B). We also observed staining with the *Rop* probe of cell clusters in the peripheral nerve system (not shown). In stage 14 (10.3 to 11.3 hours) the *Ras2* probe marked, in addition to the garland cells, a specific set of cells in the central nervous system (CNS; Fig. 4C,E). The *Rop* probe also detected the garland cells. A stronger signal in the CNS was observed, probably due to the increase in the total number of cells found in this region (Fig. 4D,F). In stages 15 and 16 (11.3 to 13 hours and 13 to 16 hours, respectively), the *Rop* probe faintly stained all peripheral nerve cell clusters (data not shown, Fig. 4J,L). In stage 17 (16 hour embryos) the CNS is condensed towards the head. In this stage, there was a strong and general staining of the CNS by the *Rop* probe. Except for several clusters in the brain hemispheres, most other cells were stained. In the ventral nerve cord, the central cells stained stronger than the outer cells. Strong staining was also observed in the antennal-maxillary complex (Fig. 4N,O). This complex was also stained by the *Ras2*

A



B



Fig. 2. Sequence alignments of the Rop protein. (A) Homology of Rop with the *S. cerevisiae* Sly1p and Sec1p proteins. Identical residues are boxed in black, similar residues are boxed in grey. The percentages of the identical/similar amino acids between these genes are 25/38, 24/40 and 21/34 for Rop and Sly1p, Rop and Sec1p, Sly1p and Sec1p, respectively. (B) Alignment of a conserved motif among yeast, *Drosophila* and rat proteins. The following amino acids were considered similar: L,I,V,M; G,A,S,T,P; D,E,Q,N; K,R,H; W,Y,F.

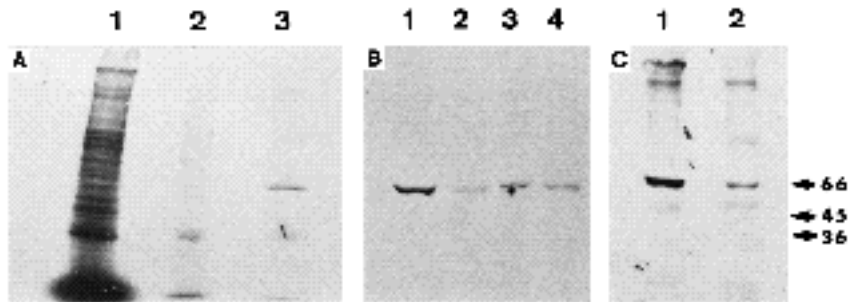


Fig. 3. The *Rop* gene product. (A) Immunoprecipitation of the *Rop* protein from S2 cells grown with [³⁵S]methionine. 1, Total proteins; 2, immunoprecipitation with non-immune serum; 3, immunoprecipitation with anti-*Rop* serum. (B) Immunoblot of total proteins from Schneider S2 cell lines reacted with anti-*Rop* antibody. 1, 2, S2 cell line stably transformed with an *hsp70-Rop*

transcription fusion; 3, 4, normal S2 cell line; 1, 3, with heat shock; 2, 4, without heat shock. (C) Detection of the *Rop* protein in adult flies. Immunoblot of total proteins from 1, males; 2, females, reacted with anti-*Rop* antibody.

(Fig. 4N,O). This complex was also stained by the *Ras2* probe, but to a lesser extent (Fig. 4M).

Activity of the *Ras2/Rop* bidirectional promoter in the larva and the adult fly

To learn about the expression of the two genes in postembryonic stages, the coding region of the bacterial *lacZ* gene was attached to the bidirectional promoter in either the *Ras2* orientation or the *Rop* orientation. The *lacZ* gene was pro-

vided by the vector pW-ATG-*lacZ* (Kuhn et al., 1988) derived from the pW8 transducing vector (Klemenz et al., 1987), which enables the transduction of foreign DNA into cells of the *Drosophila* germ line (Spradling, 1986). The maps of the resulting transcription fusions are shown in Fig. 6. In the *Ras2-lacZ* construct, the cloned fragment spanned 78 bases downstream to the *Ras2* transcription start site and 852 bases upstream to this site. In the *Rop-lacZ* construct, the cloned fragment contained 48 bases downstream to the

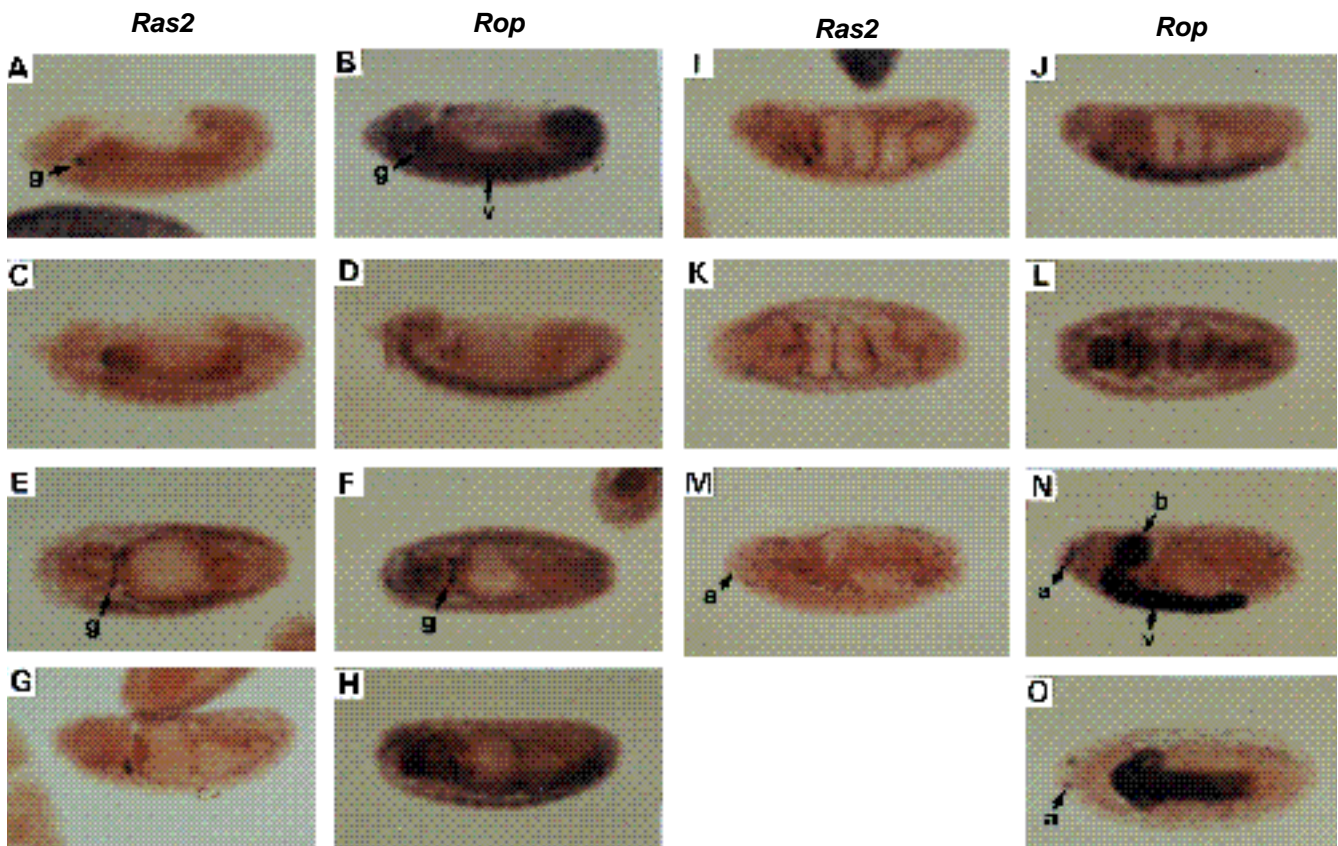


Fig. 4. Spatial distribution of *Ras2* (left column) and *Rop* (right column) transcripts during embryogenesis. In situ hybridization on whole-mount embryos with digoxigenin-labeled DNA probes. Orientation of embryos is anterior left, dorsal up. (A,B) Stage 13 and (C,D) stage 14. The *Ras2* transcripts are detected in the garland cells. *Rop* transcripts are detected in the garland cells and in the CNS. (E,F) Stage 14, a dorsal view. Abundant expression of *Ras2* and *Rop* in the garland cells, organized as a ring around the esophagus. (G,H) Stage 15, (I,J) stage 16 and (K,L) stage 16, dorsal view. The transcript localization is similar to the previous stages. (M,N) Stage 17 and (O) stage 17, dorsal view. *Ras2* transcripts are detected in the antennal-maxillary complex. *Rop* transcripts are located in the CNS and in the antennal-maxillary complex. a, antennal-maxillary complex; b, brain; g, garland cells; v, ventral nervous cord.

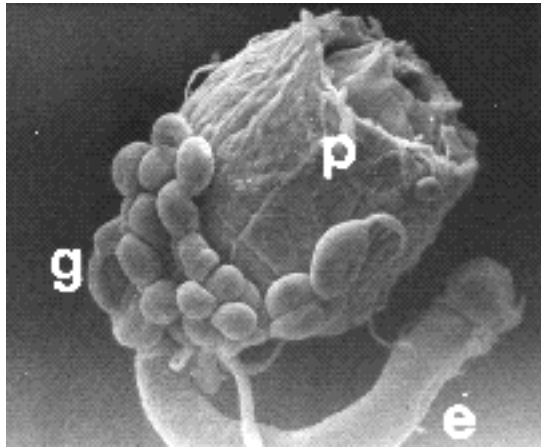


Fig. 5. Scanning electron micrograph of the garland cells. A third instar larva was dissected and a portion of the digestive system was isolated. e, Oesophagus; p, proventriculus; g, garland cells. Magnification $\times 350$.

Rop transcription start site and 1047 bases upstream to this site. Since the length of the bidirectional promoter sequence between the two transcription start sites is 93 bases, the total length of the overlapping *Drosophila* sequences between these two constructs is 219 bases (Fig. 6).

Several transgenic lines were obtained and more lines were derived by transposing X-linked transposons to the autosomes as described by Cooley et al. (1988). In summary, six *Ras2-lacZ* lines and seven *Rop-lacZ* lines were established. The authenticity of the staining pattern may be distorted by the interaction of endogenous transcription elements in the vicinity of the transposon integration site with the transcription control elements of the *Ras2* and *Rop*

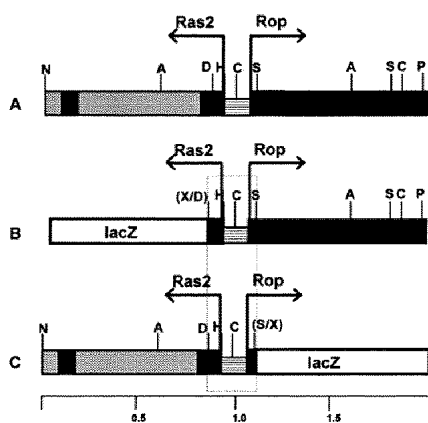


Fig. 6. Schematic maps of the *Ras2-lacZ* and *Rop-lacZ* transcription fusions. (A) The native sequences of the *Ras2/Rop* region. (B) The *Ras2-lacZ* construct. (C) The *Rop-lacZ* construct. Transcription start sites and gene polarity are marked with arrows. Filled bar, exon; grey bar, intron; striped bar, the bidirectional promoter region; empty bar, the *lacZ* reporter gene. The dashed box designates the *Drosophila* sequences common to the two constructs. Scale is in kb. Restriction sites: A, *Ava*I; C, *Cla*I; D, *Dra*I; H, *Hpa*I; N, *Hind*III; P, *Pst*I; S, *Sal*I; X, *Xba*I.

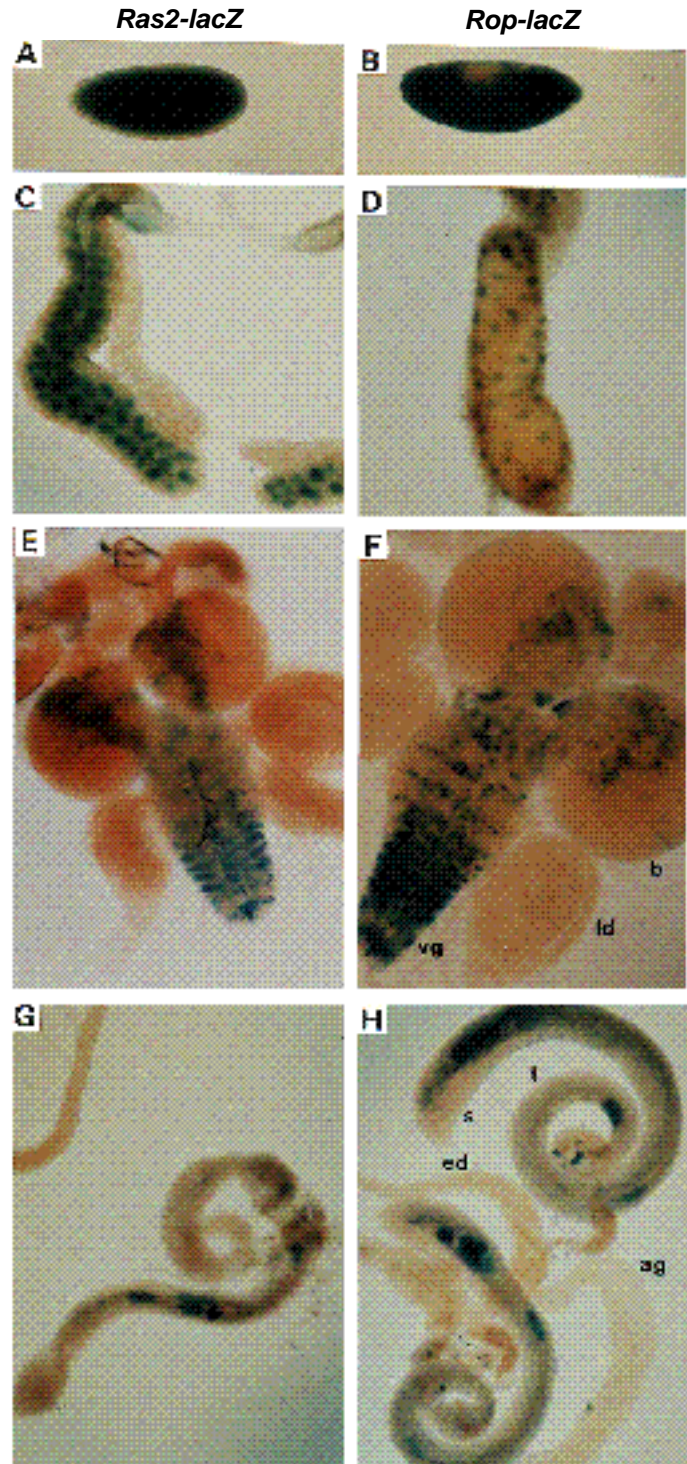


Fig. 7. Transcriptional activity of the *Ras2/Rop* bidirectional promoter in the larva and the adult fly. Similar patterns of β -galactosidase activity determined by X-gal staining of dissected organs were obtained in *Ras2-lacZ* (left column) and *Rop-lacZ* (right column) transgenic lines. (A,B) embryos; (C,D) salivary glands; (E,F) larval CNS; b, brain; ld, leg imaginal disc; vg, ventral ganglion; (G,H) testes; ag, accessory gland; ed, ejaculatory duct; s, spermatogonium; t, testis.

genes included in the transposon. Other position effects may be harmful and misleading as well. To compensate for possible interferences, several lines were analyzed simultaneously. Although some variation was observed regarding the signal intensity, the unique characteristics of the different staining patterns were the same in all cases.

The number of tissues and organs tested was limited to those that can be isolated by dissection and which do not possess high endogenous β -galactosidase activity. Embryos were stained homogeneously in almost all lines: this is probably due to the maternal contribution of the fusion-encoded β -galactosidase, which is a relatively stable enzyme (Fig. 7A,B). We tried to use transgenic males and wild-type females in order to overcome the masking of zygotic expression by the maternal contribution, but the signal was too weak to be detected either by X-Gal staining or by using anti- β -galactosidase antibodies. In the larval salivary glands, all cells were stained (Fig. 7C,D). A highly specific staining pattern was obtained in the larval CNS, which was identical in the *Ras2-lacZ* and *Rop-lacZ* lines tested (Fig. 7E,F). Specific cells within the ventral ganglion and the brain hemispheres were distinctly marked. Apparently the staining was limited to ganglion mother cells and larval nerve cells that had already terminated their differentiation and were not dividing anymore. The neuroblasts, which undergo many divisions in the brain hemispheres to form the adult optic centers, were not stained. The staining of the larval ventral ganglion was segmental. The three lines of the thoracic ganglia and the eight lines of the abdominal ganglia were clearly observed. Several types of imaginal discs, including the eye-antenna discs, the wing discs, the haltere discs and the genital discs, were also included in these preparations. However, none of them showed β -galactosidase activity originating at the transposons.

A specific staining pattern was also obtained in the adult testis. It was similar in the *Ras2-lacZ* and *Rop-lacZ* lines. The spermatogonia, which are the parental sperm cells, undergo four divisions which give rise to immature cysts containing 16 nuclei. There is no β -galactosidase expression in this stage. However, after a while the growing cysts get larger and transcription commences. At this stage, strong activity of β -galactosidase was observed in the maturing cysts, in all the *Ras2-lacZ* and *Rop-lacZ* lines tested (Fig. 7G,H). No activity was found in the accessory gland nor in the ejaculatory duct. In the female reproductive system, the X-gal staining was apparent in all cells, including the nurse cells, the follicle cells and the oocytes. Mature oocytes frequently stained more intensely (not shown).

DISCUSSION

Co-expression of the *Ras2* and *Rop* genes during early *Drosophila* development

The extent of the expression of the *Ras2* and *Rop* genes during *Drosophila* development was initially studied by poly(A)-RNA blotting analysis (Lev et al., 1985; Mozer et al., 1985). Apparently the two genes are expressed in unfertilized eggs, embryos, larvae, pupae and adult flies. The level of expression of the *Ras2* 1.8 kb transcript is fairly

constant during all stages. A shorter transcript is evident in unfertilized eggs and early embryos. The expression of the *Rop* transcripts is less uniform. In unfertilized eggs and early embryos it is low, increasing in older embryos and young larvae. In mature larvae it is low again, increasing strongly in pupae and adult flies. In all stages, the 2.2 kb transcript is more prevalent than the longer, 2.6 kb transcript. However, studies utilizing in situ hybridization to tissue sections revealed that, except for the embryo, in which a uniform, homogeneous pattern of hybridization was found, *Ras2* expression in the larva and the adult fly was limited to a number of tissues and organs (Segal and Shilo, 1986).

Our results, using in situ hybridization to whole embryos, suggest that the transcript distribution of the *Ras2* gene is differentially regulated in the embryo as well (Fig. 4). The first indication for specific *Ras2* localization is seen during germ band retraction (stages 12-13) in the garland cells. *Rop* is also detected in the garland cells and, in addition, in the CNS. At this stage, the neuroblasts start to differentiate into neurons (Campos-Ortega and Hartenstein, 1985). There are no signs of *Rop* expression in the neurogenic region in the earlier stages of CNS development, namely stages 8-10, in which neuroblasts are segregating from the neuroectoderm. This timing suggests that *Rop* may have no role in the differentiation of the neuroblasts but rather in their function as neurons. During all later stages, the *Ras2*-specific staining is limited to a specific set of cells in the CNS (weak signal) and to the garland cells (strong signal). However, *Rop*-specific staining gets stronger as the number of cells in the CNS increases. Except for several clusters in the brain hemispheres whose nature is unknown, the staining seems to be strong and general in all cells of the CNS. In the peripheral nervous system, the staining is weak, but appears in all neurogenic clusters. Distinct co-expression of both genes in the antennal-maxillary complex is also evident. Staining of the CNS and the garland cells was also observed using anti-*Rop* antibodies (N. H., unpublished results).

Co-expression of the *Ras2* and *Rop* genes during postembryonic development

In previous in situ hybridization experiments, *Ras2* transcripts were detected in the larval imaginal discs, but not in the salivary glands (Segal and Shilo, 1986). Additionally, *Ras2* transcripts were detected in the cortex of the larval brain, but not in its interior. In adult flies, specific hybridization was found in the brain cortex, the thoracic and abdominal ganglia, the flight muscles and the ovaries.

We have used *Ras2*- and *Rop*-promoter fragments fused to a β -galactosidase reporter gene to detect *Ras2* and *Rop* expression in postembryonic stages (Fig. 7). Our results show that both genes are potentially co-expressed in the larval salivary glands and CNS, and in the adult CNS and reproductive systems. The utilization of a reporter gene to detect promoter activity is a widely used approach, providing unsurpassed resolution and sensitivity. However, in the fusion constructs, any putative control elements upstream of the promoter fragment and downstream of the fusion junction are missing. Consequently, a false signal may be obtained, or a real signal may be absent, due to the

lack of an essential negative or positive control element, respectively. For example, in situ hybridization of the imaginal discs with a *Ras2* probe indicated that this gene is expressed in these organs (Segal and Shilo, 1986). However, we were not able to detect this expression using the reporter gene approach (Fig. 7E,F).

It is possible that our negative results in this case are due to weak activity of the transposon-encoded β -galactosidase. Alternatively, a positive enhancer, specific for *Ras2* expression in the imaginal discs, may be missing in our *Ras2-lacZ* fusion. Nevertheless, in those cases where positive β -galactosidase activity was detected, it is clear that the *Ras2*- and *Rop*-promoter fragments tested have at least the potential to promote the identical tissue-specific patterns as illustrated in Fig. 7. It is very likely that this potential is realized, since anti-Rop antibodies detected *Rop* protein in extracts of larval and adult brains (A. S., unpublished results). Furthermore, using in situ hybridization with the *Rop* probe, the highly specific pattern of β -galactosidase activity obtained in the larval ventral ganglion and brain hemispheres was reproduced by the pattern of *Rop* transcript localization in these organs (N. C., unpublished results).

Why a bidirectional promoter?

The two promoter fragments examined in these studies are overlapping along a sequence of only 216 bases (Fig. 6), which contains the 93 bases of the bidirectional promoter region between the two start sites and 123 bases from the untranslated regions of the two genes. Since both promoter fragments give rise to the same specific patterns shown in Fig. 7, we are forced to conclude that these patterns are determined by the same set of control elements located within this short overlapping region. We have already shown that all the *cis*-acting elements required for the activity of the bidirectional promoter in both orientations, in Schneider 2 tissue culture cells (Lev et al., 1989) and in growing embryos (N. C., unpublished results) are located within the 93 base-pair region between the *Ras2* and *Rop* transcription start sites.

In general, eucaryotic genes are widely dispersed within the genome and pairs of closely located, divergently transcribed genes which are controlled by joint bidirectional promoters are infrequent. A bidirectional promoter may set strict limitations on the optimal evolution of the transcriptional control mechanism of each member of the gene pair. Any change in favor of the regulation of one gene may also be in favor of the control mechanism of the second gene, or at least not hamper it. Good reasons to keep this type of promoter organization after all are: (a) participation of the two gene products in the same regulatory or enzymatic pathway, e.g. the SV40 early and late genes (Gidoni et al., 1985) and the yeast GAL1 and GAL10 genes (Giniger et al., 1985); (b) participation in the same biological structures, e.g. a pair of H2A and H2B sea urchin late histones (Kemler and Busslinger, 1986), the human collagens

1(IV) and 2(IV) (Burbelo et al., 1988) and (c) co-expression of the two genes in one common tissue e.g. the *Drosophila* glue proteins *sgs7* and *sgs8* (Hofmann et al., 1991), the *Drosophila* yolk proteins *yp1* and *yp2* (Logan et al., 1989), the *B. mori* chorion proteins 2132 and 2574 and

other chorion gene pairs (Iatrou and Tsitilou, 1983). Apparently, the results presented in Figs 4 and 7 suggest that the *Ras2* and *Rop* genes are actually co-expressed in several *Drosophila* tissues. Although other possibilities cannot be excluded, it is very likely that it is the simultaneous demand for the *Ras2* and *Rop* gene products in at least one of these tissues that has kept their bidirectional promoter in its unusual compact organization.

The putative role of the *Rop* gene

The sequence homology between *Rop* and Sly1p, Sec1p and Vps33p, the three yeast proteins involved in vesicle trafficking and the conserved motif that they share with β -COP, suggests that the *Rop* protein may also function in vesicle trafficking among membranes of *Drosophila* cells. It has been shown that both β -COP, which cycles between the cytosol and the Golgi, and Sly1p can be found in the cytosolic and membrane subcellular fractions (Duden et al., 1991; Donaldson et al., 1991; C. Dascher, personal communication). Likewise, the *Rop* protein can be either soluble or bound to membranes (A. S., unpublished results).

The tissue-specific expression of the *Rop* gene during embryogenesis in the CNS, a tissue that is highly active in membrane recycling, and particularly in the garland cells, is in concert with this suggestion. The garland cells, also termed wreath cells, surround the esophagus near its junction with the proventriculus (Fig. 5; Rizki, 1978). The cells are already differentiated in the embryo and they persist to the adult stage. Studies bearing on the ultrastructure of these cells reveal extensive invaginations of the plasma membrane, termed the labyrinthine channels, which are the origin of a number of coated pits and coated vesicles. Many vacuoles are found in this region. The garland cells are considered to be accessory cells to the open blood system and have been implicated in the removal of waste materials from the haemolymph by endocytosis (Aggarwal and King, 1967; Narita et al., 1989).

The putative role of the *Ras2* gene

Considering the relatively high expression of *Ras2* in the garland cells and its putative interaction with *Rop* (as discussed below), it is possible that *Ras2* is also involved in endocytic processes and/or other transport pathways mediated by vesicle trafficking. The role of yeast small GTPases in these pathways has been mentioned above. In vertebrates, a large number of ras-like proteins are involved in exocytosis and endocytosis (e.g. Chavrier et al., 1990). Two of these proteins, rab4 and rab5, have recently been implicated in the regulation of distinct early events in the endocytic pathway (Bucci et al., 1992; van der Sluijs et al., 1992).

Studies with the activated form of the *Ras2* protein suggest that it may also be associated with developmental processes. Expression of *Ras2* protein, in which the glycine at position 14 (which corresponds to glycine at position 12 of the human Ha-ras) was mutated in vitro into valine, resulted in a variety of phenotypic changes (Bishop and Corces, 1988). These changes were observed either in ectopic expression under the regulation of the *hsp70* gene promoter, or in normal expression under the regulation of the endogenous *Ras2* promoter. They include low fertility,

developmental disturbances in the wings and bristles of variable size and placement. A dual role for a small GTP-binding protein in vesicle transport and in the control of cell proliferation has already been documented. Microinjection of human *Ha-ras* protein into rat embryo fibroblasts resulted in increased membrane ruffling and fluid phase pinocytosis (Bar-Sagi and Feramisco, 1986).

Possible interaction between the *Ras2* and *Rop* proteins

Our finding that the *Rop* protein is homologous to the products of the *S. cerevisiae* *SLY1* and *SEC1* genes is very stimulating, since the *SLY1-20* allele was isolated in a screen designed to identify suppressors of the loss of the *YPT1* gene function (Dascher et al., 1991; Ossig et al., 1991). *YPT1* is a *ras*-like gene (Gallwitz et al., 1983) essential for ER-to-Golgi protein transport and possibly also participates in intra-Golgi vesicle movements (Schmitt et al., 1988; Segev et al., 1988). The *SLY1-20* allele, which allows *YPT1*-independent growth, contains a single point mutation in the wild-type *SLY1* gene (Dascher et al., 1991). The mutation is absolutely required to complement the loss of *YPT1*, since overexpression of the wild-type *SLY1* allele is not sufficient for complementation. A possible mechanism of the suppression is that *SLY1* encodes a protein acting downstream of *YPT1*. It is suggested that the mutated Sly1-20p protein has a specific conformation which the wild-type protein can achieve only through interaction with Ypt1p (Ossig et al., 1991). Thus, *SLY1* may interact, directly or indirectly, with *YPT1*.

Interestingly, *SEC1* and *SEC4*, a *ras*-related gene (Salminen and Novick, 1987), may be another yeast gene pair of a similar type, since both genes are involved in protein transport from the Golgi to the plasma membrane. However, in this case there is as yet no proof of genetic or biochemical interaction between them. Finally, γ -COP also interacts with a member of the *ras* superfamily. Recently it has been shown that ARF, a *ras*-like ADP ribosylation factor, is required for the binding of γ -COP to Golgi membranes (Donaldson et al., 1992). Thus, the Rop/Sly1p/Sec1p/ γ -COP sequence homology may represent not only common sites associated with transient binding to internal membranes but also common sites required for their interaction with small GTP-binding proteins.

As discussed above, the unusually close proximity of the *Ras2* and *Rop* genes and their joint regulative mechanism could be due solely to the need for their products in the same tissue. However, since Sly1p, Sec1p and γ -COP, may interact with small GTP-binding proteins of the *ras* superfamily, it is conceivable that the *Rop* and *Ras2* proteins may also interact functionally with one another in these tissues.

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