A Novel Drosophila Alkaline Phosphatase Specific to the Ellipsoid Body of the Adult Brain and the Lower Malpighian (Renal) Tubule

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Manuscript received June 15, 1999
Accepted for publication September 20, 1999

ABSTRACT

Two independent Drosophila melanogaster P[GAL4] enhancer-trap lines revealed identical GAL4-directed expression patterns in the ellipsoid body of the brain and in the Malpighian (renal) tubules in the abdomen. Both P-element insertions mapped to the same chromosomal site (100B2). The genomic locus, as characterized by plasmid rescue of flanking DNA, restriction mapping, and DNA sequencing, revealed the two P[GAL4] elements to be inserted in opposite orientations, only 46 bp apart. Three genes flanking the insertions have been identified. Calcineurin A1 (previously mapped to 21E-F) lies to one side, and two very closely linked genes lie to the other. The nearer encodes Aph-4, the first Drosophila alkaline phosphatase gene to be identified; the more distant gene [(3)9601] is novel, with a head-elevated expression, and with distant similarity to transcription regulatory elements. Both in situ hybridization with Aph-4 probes and direct histochemical determination of alkaline phosphatase activity precisely matches the enhancer-trap pattern reported by the original lines. Although the P-element insertions are not recessive lethals, they display tubule phenotypes in both heterozygotes and homozygotes. Rates of fluid secretion in tubules from c507 homozygotes are reduced, both basally, and after stimulation by cAMP, or Drosophila leucokinin. The P-element insertions also disrupt the expression of Aph-4, causing misexpression in the tubule main segment. This disruption extends to tubule pigmentation, with c507 homozygotes displaying whitelike transparent main segments. These results suggest that Aph-4, while possessing a very narrow range of expression, nonetheless plays an important role in epithelial function.

ALKALINE phosphatase (ALP) is a zinc and magnesium-containing metalloenzyme (EC 3.1.3.1) that hydrolyzes phosphate esters with a high pH optimum. It is found in most species from bacteria to humans. In Escherichia coli, ALP (encoded by the gene phoA) is found in the periplasmic space. In yeast, ALP (encoded by the gene PHO8) is found in lysosome-like vacuoles. And in mammals, it is a glycoprotein attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor (McComb et al. 1979; Trowsdale et al. 1990).

In mammals, four different ALP isozymes are currently known, each encoded by at least one separate gene. Three are tissue specific: the placental, placental-like (germ cell), and intestinal isozymes. The fourth form, previously known as the liver/bone/kidney isozyme, is tissue nonspecific and has physical and biochemical properties that clearly distinguish it from the other ALP isozymes (Harris 1989; Manes et al. 1990).

There are also several independent ALP loci in Drosophila melanogaster (Yao 1950; Beckman and Johnson 1964; Schneiderman et al. 1966; Harper and Armstrong 1972, 1973; Parkash et al. 1993), but none has been previously characterized at the molecular genetic level.

The enhancer-trap technique has proved valuable for identifying and isolating genes with particular spatial or temporal patterns of expression (O'Kane and Gehring 1987; Bier et al. 1989; Wilson et al. 1989). We have recently used the P[GAL4] enhancer-trap system (Brand and Perrimon 1993) to generate and screen over 1400 independent Drosophila lines for patterns of GAL4-directed β-galactosidase expression in the adult brain (Yang et al. 1995) and in the Malpighian (renal) tubules (Sözen et al. 1997). Two lines with identical expression characteristics (c507 and c232) were selected by both criteria and analyzed in detail. Expression in the brain is limited to only a small subset of neurons belonging to the ellipsoid body of the central complex, a structure implicated in the coordination of motor activity (Strauss and Heisenberg 1993; Illius et al. 1994). Expression in the Malpighian tubules is restricted to a zone associated with fluid reabsorption (O'Donnell and Maddrell 1995; Sözen et al. 1997).

Here we report the characterization of the genomic region encompassing the P[GAL4] elements in lines c507 and c232. We find the expression patterns reported by their LacZ enhancer detectors to correspond to that of a nearby ALP gene (Aph-4), implying a defined role...
for this isozyme in specific aspects of neural and renal function.

**MATERIALS AND METHODS**

**Drosophila strains**: P[GAL4] lines described here were isolated in our laboratory (Yang et al. 1995). The secondary reporter for GAL4 activity was a second chromosome insertion of UAS_{lacZ} [Br and Perrimon 1993]. There is no visible β-gal expression in the absence of GAL4. P[AcW] lines were kindly provided by D’Evelyn et al. (1998) and P[PF] line l(3)07028 was kindly provided by A. Spradling’s laboratory. All genetics markers used in the work were described by Lindsey and Grel (1968). Flies were maintained on standard cornmeal/yeast/agar medium at 25°C (Ashburner 1989).

**Histology**: To obtain sections, recombinant flies carrying P[GAL4] and P[UAS_{lacZ}] were mounted in “fly collars” (modified from Heisenberg and Bohr 1979), soaked in OCT embedding medium (Miles, Kankakee, IL) for 10 min and then embedded in the OCT medium. Twelve-micrometer serial sections of head or body were cut in a cryostat (Anglia Scientific) at −18°C. The sections were stained and mounted as described by Yang et al. (1995). Thereafter, sections were examined and photographed on a Nomarski optical microscope.

For whole-mount preparations, brains or Malpighian tubules were dissected in PBS and fixed in 4% paraformaldehyde for 20 min. They were then washed three times for 20 min in PBS and stained with staining buffer and 2% X-gal for 1-2 hr at 37°C. They were then washed for 20 min in PBS, cleared overnight at 4°C with PBS/12.5% hydrogen peroxide, washed for 10 min with PBS, dehydrated through graded ethanol, and mounted in glycerol gelatin.

For histochemical detection of alkaline phosphatase activity, brains or Malpighian tubules were dissected in PBS and fixed in 4% paraformaldehyde for 20 min. They were then washed three times for 20 min in PBS and stained with NBT/ X-phos in digoxigenin (DIG) detection buffer for up to 1 hr (Boehringer Mannheim). Heads and bodies of wild-type flies were cut into 12-µm sections using a cryostat (Yang et al. 1995) and hybridized with the relevant probe.

**Assay of tubule secretion phenotype**: Tubules were dissected from adult (3-7-day-old) flies and placed in 10 µl drops of Schneider’s medium: Drosophila saline under paraffin oil. Fluid secretion experiments were performed as previously described (Dow et al. 1994). The reaction was stopped by washing in PBT and mounted in glycerol gelatin (Sigma, St. Louis).

**Molecular methods**: Genomic sequences flanking the P[GAL4] element were cloned by plasmid rescue using standard techniques (Bier et al. 1989; Wilson et al. 1989). Briefly, genomic DNA was digested with PstI to clone sequences “downstream” of the P[GAL4] element and with KpnI to clone sequences “upstream” of the P[GAL4] element, followed by ligation to form a plasmid, which was propagated in E. coli NM621. The plasmids were used to screen a Drosophila genomic DNA library in the vector EMBl3 (K. Kaiser, unpublished results), and a head cDNA library in the vector NM1149 (Russell 1989). The 5' rapid amplification of cDNA ends (RACE) procedure was carried out using Superscript RT (Life Technologies) according to the conditions recommended by the manufacturer.

**RESULTS**

Double-stranded sequencing reactions using the dyeoxy chain termination method were carried out as described in the Sequenase Version 2.0 manual (United States Biochemical Corp., Cleveland). The nucleotide sequence of both strands was obtained for all transcribed regions. Intron sequences were sometimes read on one strand only. DNA sequences were analyzed using MacVector and AssemblyLIGN (Sequence Analysis Software), BLAST and Prosite (NCBI), GCG (Wisconsin), and SeqVu (Garvan Institute).

Total Drosophila RNA was isolated using the acidic guanidinium thiocyanate method (Chomczynski and Sacchi 1987). Poly(A)⁺ RNA was selected by oligo(dt) chromatography, separated in 1% 3-[N-morpholino]propane-sulfonic acid (MOPS)/formaldehyde denaturing gels, transferred to nitrocellulose, and probed with radiolabeled cDNA fragments. Hybridization was carried out at 42°C in 50% formamide, 5x SSPE, 0.04% bovine serum albumin/0.04% polyvinylpyrrolidone/0.04% Ficoll/0.1% sodium dodecyl sulfate (SDS). These filters were then stripped and reprobed with rp⁴ (O’Connell and Rosbach 1984) as a loading control.

**In situ hybridization**: The procedure for in situ hybridization to tissue sections was carried out essentially as described by Pardee (1986). pBluescript and cDNAs were labeled with Bio-16-dUTP by nick-translation. Hybridization was detected using 3'-3-diaminobenzidine tetrahydrochloride (DAB)/ H₂O₂. After hybridization, the slides were stained with Giemsa and mounted using DPX mountant.

Nonradioactive in situ hybridization to tissues was carried out essentially as described by Nighorn et al. (1991). Relevant cDNA fragments were used as templates for the synthesis of DIG-labeled single-strand RNA probes according to the manufacturer’s instructions (Boehringer Mannheim). Heads and bodies of wild-type flies were cut into 12-µm sections using a cryostat (Yang et al. 1995) and hybridized with the relevant probe.

Hybridization signals were detected by two different methods. (1) Colorimetric detection with NBT and X-phosphate. For immunological detection of the hybridized probes, the sections were washed in PBT and incubated with 200 µl of 5% (v/v) sheep serum in PAT for 2-3 hr. The sections were then incubated with 150-200 µl of anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) diluted at 1:500 in PAT for 2-3 hr at room temperature or overnight at 4°C. The sections were washed twice in PBT. After extensive washes in 100 mm Tris-HCl, pH 9.5, 100 mm NaCl, 50 mm MgCl₂, and 2 mm levamisole, sections were placed with 200 µl of diluted chromogenic substrate solutions (NBT and BCIP, X-phosphate) following the manufacturer’s instructions (Boehringer Mannheim), and incubated in the dark at room temperature for 2-4 hr. The reaction was stopped by washing in PBT for 20 min and preparations were mounted in glycerol gelatin (Sigma). (2) Colorimetric detection with DAB and H₂O₂. Sections were washed in PBT and incubated with 200 µl of 5% (v/v) sheep serum in PAT for 1-2 hr. The were then incubated with 150-200 µl of anti-digoxigenin-horseradish peroxidase (anti-DIG-HRP) Fab fragments (Boehringer Mannheim), diluted at 1:500 in PAT for 2-3 hr at room temperature or overnight at 4°C. After extensive washes, signal was detected with diaminobenzidine and hydrogen peroxide according to the manufacturer’s conditions (Boehringer Mannheim). Reactions were stopped by washing in PBT for 20 min and preparations were mounted in glycerol gelatin (Sigma).

**lacZ expression patterns in P[GAL4] lines c507, c232, and P[PZ] line l(3)07028**: Lines c232 and c507 report
identical, and extremely specific, expression patterns. GAL4-directed β-gal expression in the adult occurs in just the two cellular domains shown in Figure 1, a and b. Expression in the brain corresponds to ring neurons of the ellipsoid body, and more specifically to the R3 and R4 morphological subtypes (Hanesch et al. 1989; Renn et al. 1999). Neurites arising from lateral cell bodies (CBs) extend dendritic arbors within a region known as the lateral triangle (LTR) and send axonal process toward the midline where in combination they contribute to the characteristic ellipsoid body structure. In addition, both GAL4 lines mark the lower section and ureter of the Malpighian tubules (Figure 1, d and e), regions associated with reabsorption of fluid from the primary urine (O’Donnell and Maddrell 1995; Sözen et al. 1997).

In P[PBZ] line l(3)07028, β-gal expression is seen in the nuclei of cells in the lower tubules and ureter (Figure 1g), as for the two GAL4 lines. No staining is seen in the adult brain, however. By in situ hybridization to polytene chromosomes, both P[GAL4] insertions were found to reside at the same cytological location in the proximal part of 100B (Figure 2), consistent with the localization of 100B2 documented for line l(3)07028 [Bloomington Drosophila Genome Project (BDGP) accession no. AQ073337].

**Figure 1.**—Expression patterns in enhancer-trap lines and wild-type flies. (a and b) LacZ staining of ellipsoid body ring neurons in the adult brain of line c507. (c) Analogous section of a wild-type head showing Aph-4 mRNA expression detected by the standard anti-DIG method. (d and e) LacZ staining of the lower Malpighian tubule and ureter in line c507. (f) Analogous section of a wild-type body showing coexpression of Aph-4 RNA as detected by the standard anti-DIG method in the presence of levamisole. The lower right inset shows the hybridization pattern seen using the DAB/peroxidase detection system. (g) LacZ reporter staining of the lower Malpighian tubule and ureter in semilethal line l(3)07028. (h) Or region R, showing wild-type natural pigmentation pattern of opaque main segments and lower tubules. (i) c507 homozygotes, displaying transparent main segments (reminiscent of w− mutants) and opaque lower tubules. (j) Wild-type tubule alkaline phosphatase activity is concentrated exclusively in the ureter and lower tubule. (k) Alkaline phosphatase activity is concentrated in the main segment of the tubule in homozygous c507 flies. (l) Alkaline phosphatase activity is concentrated in the ureter, lower tubule, and main segment in heterozygous c507 flies. CB, ring neuron cell bodies; LTR, lateral triangle; EB, ellipsoid body; LT, lower Malpighian tubule; U, ureter; M, main segment. Line c232 gives identical staining patterns.

**Plasmid rescue of flanking genomic DNA from lines c507, c232, and l(3)96601:** The enhancer-trap element contains a bacterial plasmid replicon flanked by unique restriction sites, permitting rescue of genomic DNA from either side of the insertion by appropriate choice of enzyme (Wilson et al. 1989). Genomic DNAs from P[GAL4] lines c507 and c232 were digested with PstI, ligated, and rescued to produce downstream (i.e., 3′ to GAL4 transcription unit) plasmids pPC507 and pPC232, respectively. Similarly, KpnI was used to obtain the upstream (i.e., 5′ to GAL4 transcription unit) plasmid pKC507 from line c507 (Figure 3). In similar fashion, genomic DNA from P[facW] line l(3)96601 was digested with EcoRI or PstI to produce downstream plasmid pE96601 and upstream plasmid pP96601 (Figure 3).

Although the P[GAL4] elements in lines c232 and c507 map to the same polytene band and give rise to identical expression patterns, the downstream rescued fragments are of different size. To examine further the relationship between the two insertions, restriction mapping and DNA sequencing analysis was performed, revealing the two P[GAL4] elements to be inserted 46 bp apart and in opposite orientation (Figures 3 and 4).

Rescued fragments from line c507 were used as probes to screen an EMBL3 genomic DNA library, resulting in three different classes of inserts (λ1−λ3).
These were arranged into a contig by restriction mapping and Southern blot analysis (Figure 3).

### Isolation of cDNA clones
The rescued fragment from pPC507 contains a 0.9-kb BamHI fragment, which, on the basis of "reverse Northern" analysis (data not shown), appeared to be transcribed. The 0.9-kb fragment was thus used to probe a Drosophila head cDNA library. Two independent (non-cross-hybridizing) classes of cDNA were obtained, the longest versions of which were designated pMY51 and pMY8 (Figure 3). They hybridize to nonoverlapping regions of the genome as shown in Figure 3. The head cDNA library was also probed with a 4.5-kb HincII fragment from pKC507. The longest cDNA was designated pMY4 (Figure 3).

In situ hybridization to wild-type polytene chromosomes with each of the above three cDNAs showed the respective transcription units to be contained within the 100B2 region (Figure 2, left). Southern blotting further confirms a single locus for each of the respective genes (Figure 2, right).

**pMY51 encodes an alkaline phosphatase:** Figure 4 shows the nucleotide and deduced polypeptide sequences of the pMY51 insert and of the 5' RACE product derived from pMY51 cDNA. The equivalent region of genomic DNA was also completely sequenced, showing the gene to be punctuated by three introns of 1332, 67, and 60 bp, respectively. The 1952 bp cDNA contains a long open reading frame (ORF) of 578 amino acids. Database searching showed the deduced polypeptide to be clearly a member of the ALP family (see Figures 5 and 6) containing the highly conserved ALP signature "VPDAGTAT." Three Drosophila ALP loci have been previously described (Aph-1,-2,-3), although none has yet been cloned. The gene described here corresponds to none of the above on the basis of cytological location and thus has been designated Aph-4. Like most other ALPs, Aph-4 has five potential N-linked glycosylation signals, the positions of which vary between species (Manes et al. 1990; Itoh et al. 1991). The cDNA does not contain an obvious polyadenylation site, and none is seen in the genomic interval between Aph-4 and the 3' end of the (3)96601 transcription unit (see also Figure 3).

Figure 4 also shows the sequence of a genomic interval containing the three P-element insertion sites of P (GAL4) c507, c232, and P (PZ) (3)07028. Flies homozygous for the insertions in lines c507 and c232 are viable, whereas homozygotes of (3)07028 are described as having a semilethal phenotype (BDGP, accession no. AQ-073337). These closely spaced insertions presumably disrupt the promoter of the Aph-4 gene to varying extents.

Among ALPs for which sequence data are available, Aph-4 is most closely related to vertebrate isozymes, fractionally more so to tissue nonspecific varieties (Figures 5 and 6). The closest match was to a chicken nonspecific isoform precursor (Crawford et al. 1995) with 43% identity and 51% similarity (determined using GAP). The only other insect ALP sequence available is from the silkworm Bombyx mori (Itoh et al. 1991; 38% identity, 47% similarity), presumably a parologue. Metal and substrate phosphate binding sites determined for *E. coli* ALP by X-ray crystallography (Sowadski et al. 1985), and which are strongly conserved across phyla, are also present in Aph-4 (asterisks in Figure 5).

**Aph-4 expression:** Aph-4 mRNA abundance is highest during larval and adult stages (Figure 7, left). Such a profile parallels that described for ALP activity in a
Figure 3.—Organization of genomic interval surrounding the P\{GAL4\} insertions in lines c507 and c232. (a) Three EMBL3 genomic DNA clones. (b) Partial restriction map and sites of P-element insertion. The P elements are shown schematically and are not to scale. The bold segment of each is the plasmid replicon (3' with respect to the reporter gene at the opposite end of the P element). The P\{GAL4\} elements in lines c507 and c232 are 46 bp apart and in opposite orientations. The P\{PZ\} element in line l(3)07028 is inserted 19 bp closer to the Aph-4 gene than the c232 insertion but has the same orientation as the c507 insertion (see also Figure 4). The P element in line dbt* (doubletime) is homozygous lethal (Kloss et al. 1998). The P\{adW\} element in line l(3)96601 lies in the putative 5' untranslated region of the l(3)96601 gene. (c) Extent of rescued flanking DNAs, pKC507 to KpnI site; pPC232 and pPC507 to PstI sites; pE96601 to EcoRI site; pP96601 to PstI sites. (d) Approximate extent of cDNA clones pMY4, pMY51, pMY8, and 5' RACE product from pMY51. (e) Deduced gene structures (that of CanA1 being derived from Guerini et al. (1992) and of dbt from Kloss et al. (1998)). B, BamHI; E, EcoRI; H, HindII; K, KpnI; P, PstI; S, SalI.
Figure 4.—Nucleotide and deduced polypeptide sequences of Aph-4. Uppercase nucleotide sequences are from cDNA, lowercase from genomic DNA. The 3' end of the transcriptionally convergent l(3)96601 gene (pMY8 insert) is shown in italic type. The highly conserved ALP motif VPDSAGTAT is boxed. Sites of putative N-glycosylation are underlined. P{GAL4} insertions in lines c507 and c232 are in opposite orientation. The P{PZ} insertion in line l(3)07028 has the same orientation as the c507 insertion. The Aph-4 cDNA has been assigned the EMBL/GenBank/DDBJ accession no. X98402.
range of insects (McComb et al. 1979). A dramatic increase of Drosophila ALP activity in third instar larvae, prior to the secretion of pupal cuticle, has been taken to suggest a role for the ALP in cuticle formation, possibly regulated by the ring gland (Schneiderman et al. 1966).

In situ hybridization to sectioned adult heads and bodies revealed Aph-4 mRNA within the same cellular domains as revealed by GAL4-directed β-gal expression, i.e., the ellipsoid body (Figure 1, b and c) and the Malpighian tubules (Figure 1, e and f). Since the detection of DIG-labeled probes usually relies on an ALP reporter enzyme coupled to anti-DIG antibodies, fresh levamisole was used to block endogenous phosphatase expression (McComb et al. 1979), even though ALP activity in any case appeared to be lost during hybridization (data not shown). An independent detection system, employing diaminobenzidine/ peroxidase staining to detect a peroxidase/ anti-DIG reporter gave the same result (Figure 1f, lower right inset).

The c507 insertion disrupts normal tubule pigmentation: Normal tubules in wild-type fly (Oregon-R) have an opaque, yellowish color throughout the main segment and lower tubules (Figure 1h), caused by the accumulation of hydroxy-kynurenine and associated eye pigments, through the action of the white gene product and other organic solute transporters (Wessing and Eichelberg 1978). In c507 homozygotes, this pigmentation is strikingly suppressed in the main segment, but not in the lower tubule (Figure 1i). A possible explanation is an effect of the Aph-4 promoter on expression of the mini-white gene contained within the nearby enhancer-trap element (X chromosomes of c507 and c232 carry the dysfunctional w^{118} allele). A powerful lower tubule enhancer/ main segment repressor element might influence not just the lacZ reporter, but also mini-white, in a lower-tubule-specific pattern.

The c507 insertion causes misexpression of Aph-4 to a genetically distinct domain: We found wild-type tubule alkaline phosphatase activity to be concentrated exclusively in the ureter and lower tubule (Figure 1j), identical to the three enhancer-trap patterns (Figure 1, d and g). The Aph-4 pattern in c507 homozygotes was profoundly different, however (Figure 1k). Expression in the lower tubule is reduced compared to wild type, while that in the main segment is greatly enhanced (Figure 1, j and k). c507 heterozygotes have an intermediate pattern with expression concentrated in lower tubule and ureter, but with some expression in main segment (Figure 1i). The main segment is a genetic domain defined by Sozen et al. (1997), which is known to correspond to the fluid-secreting portion of the tubule (O'Donnell and Maddrell 1995). This suggests that the c507 insertion is impinging on a control region that specifies tubule expression domains. The fact that misexpression in the main segment corresponds so precisely to the domains described by Sozen et al. (1997) suggests that there is a combinatorial mechanism of transcription factors that normally specifies the lower tubule domain.

Fluid secretion phenotypes: Tubules from c507 homozygotes showed consistently lower basal rates of secretion than wild-type tubules (Figure 8). They also showed reduced responses to a range of agonists: the cyclic AMP, which acts on principal cells to stimulate the apical V-ATPase through intracellular nitric oxide/ cyclic GMP (Davies et al. 1997; Figure 8A); cyclic AMP, which acts on principal cells to stimulate the apical V-ATPase (O'Donnell et al. 1996; Figure 8B); and Drosophila leucokinin, which acts on stellate cells to stimulate fluid production by activating the chloride shunt conductance through intracellular Ca^{2+} (O'Donnell et al. 1998; Figure 8C). As the lower tubule does not participate in the fluid secretion assay, these results must be interpreted in terms of the misexpression of Aph-4 in c507 homozygotes to the tubule main segment. This expression may disrupt the tight coupling of metabolism and transport at either the basal or apical membranes.

pMY8 encodes an essential polypeptide of unknown function: pMY8 corresponds to a single genetic locus in the 100B2 region (Figure 2). The pMY8 insert is 897 bp long, excluding the 18-bp polyA tail remnant (Figure 9). A partial cDNA sequence from the Berkeley Drosophila Genome Project (GenBank accession no. AA696267) provided six additional residues at the 5' end. Genomic DNA sequencing shows the gene to be punctuated by two introns of 87 bp and 265 bp, the first of which lies 38 bp upstream of the putative translation start site. The sequence TATAAA is 84 bp upstream of the beginning of the poly(A) tract. This is not the most common of poly(A) addition signals, but it is used occasionally (Manley 1988). The pMY8 cDNA contains an ORF of 223 amino acids. Database searches revealed no obviously related genes, with the possible exception of three mammalian expressed sequence tags (ESTs) revealed by a TBLASTN search (GenBank accession nos. AA097076, AA098325, and R86167). The two likeliest possibilities are a transcription factor and a neuropeptide gene. The N-terminal part of the deduced protein contains a number of repeats (KPAA and similar) associated with histone-like DNA regulatory proteins, which are found in a range of organisms. The protein also contains several dibasic repeats (at aa 33-4, 130-1, and 291).

Deak et al. (1998) have generated a large collection of third chromosome recessive lethal lines by insertion of a P[acman] enhancer-trap element. Using the plasmid rescue method of Guo et al. (1996), we found two lines (96601 and 35313) with P[acman] insertions at the same nucleotide position and in the same orientation, just 5' to the pMY8 coding region (Figure 9). Both lines carry only a single P element and die as homozygotes during
the pupal-to-pharate adult stage (Deák et al. 1998). One of the lines, l(3)96601, and its corresponding rescued plasmid, p96601 (Figure 3), were chosen for further characterization.

Excision of the P element caused reversion of the lethal phenotype, verifying insertion as the cause of lethality (rather than an unlinked event elsewhere on the third chromosome). Southern blotting of l(3)96601/wt DNA revealed the expected band shift due to P{lacW} insertion (not shown). Northern blotting of wild-type mRNA revealed an adult transcript of ~1 kb that is slightly elevated in the head (Figure 7, middle). Northern blotting of l(3)96601/wt mRNA indicated a reduction of transcript abundance of 43% (normalized to rp49 loading control) compared with wild type (Figure 7, right). In situ hybridization to sections of the adult head and body revealed generalized expression throughout the organism.

pMY4 encodes calcineurin A1: pMY4 corresponds to a single genetic locus in the 100B2 region (Figure 2). The sequence of the pMY4 insert revealed it to be a partial (5′-truncated) Drosophila calcineurin A1 (CanA1) cDNA (Guerini et al. 1992). This was surprising, since CanA1 had been previously assigned to the cytogenetic location 21E-F (Guerini et al. 1992). There is no evidence from either Southern blotting or in situ hybridization to polytene chromosomes (Figure 2) that there are two such genes in the Drosophila genome. However, 21E-F and 100B are at similar positions to the telomeres of 2L and 3R, respectively, and so we suggest that 100B2 is the true location. In support of this, two recently sequenced BDGP P1 clones (GenBank accession nos. AC007975 and AC008220) that have been mapped to 100A-C contain sequence identical to the published CanA1 cDNA.

**DISCUSSION**

We have identified a number of genes flanking the P[GAL4] elements in the enhancer-trap lines c507 and c232. Three different genes located at 100B were cloned and characterized. One of them is calcineurin A1, a Ca2+/calmodulin-stimulated protein phosphatase (Guerini et al. 1992), which is located upstream of the P[GAL4] element in line c507. Two other closely linked genes are located downstream of the c507 element, Aph-4 being closest to the site of transposon insertion. Tightly linked to Aph-4, but in opposite orientation, is the essential l(3)96601 locus of unknown function. Both

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Figure 5.—Pileup of representative ALP protein sequences. Boxed residues are at least 50% conserved between the seven ALPs, shaded residues at least 80% similar. * indicates residues that participate in the active site of E. coli ALP. Drosophila Aph-4 (XM8402); chicken nonspecific isozyme precursor (U19108; Crawford et al. 1995); human nonspecific isozyme precursor (P05186; Weiss et al. 1986); human placental type 1 isozyme precursor (P05187; Knoll et al. 1988); human placental-like isozyme precursor (P10696; Millan and Manes 1988); human intestinal isozyme precursor (P09923; Henthorn et al. 1987); silk moth membrane-bound alkaline phosphatase precursor (P29523; Itoh et al. 1991).

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Figure 6.—Neighbor-joining plot showing phylogenetic relationships between representative ALPs. Protein accession numbers are shown in parentheses.

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Figure 7.—Northern blot analysis. A total of 10 μg of poly[3′-C]5′-C] RNA from the indicated stages and tissues was hybridized with the indicated cDNAs. As a control for loading, stripped filters were reprobed with rp49 cDNA (O’Connell and Rosbach 1984). E, 0–24-hr embryo; L, mixed larval instars; P, midpupal; H, adult head; B, adult body; wt, wild type; lacW, line l(3)96601.

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Figure 6. — Neighbor-joining plot showing phylogenetic relationships between representative ALPs. Protein accession numbers are shown in parentheses.
The \{GAL4\} insertions in lines c507 and c232 are homozygous viable, and there are no other obvious defects. To generate loss-of-function alleles, imprecise P-element excision has been carried out. Over 130 revertants were generated and analyzed. One line has a large deletion that removes part of the P element and flanking genomic DNA including the Aph-4, \(l(3)96601\), and the nearby dbt (double-time, Kloss et al. 1998; see Figure 3). Although flies homozygous for this deletion die at the late embryo or early larval stage, both \(l(3)96601\) and dbt are essential loci, so no conclusion can be drawn concerning Aph-4. However, a recently documented line, \(l(3)07028\) (BDGP accession no. AQ073337; see Figure 4), in which P\{PZ\} is inserted 19 bp closer to the 5' end of Aph-4 than the c232 insertion, has a semilethal phenotype. This may imply that Aph-4 is an essential, or at least an important, Drosophila gene.

Reverse genetic analysis in most model organisms is complicated by the "phenotype gap," a term that acknowledges the dearth of phenotypes for detailed functional analysis. This is particularly acute in Drosophila, where the small size of the organism militates against physiological study. However, the identification of cell-specific expression of Aph-4 in the Malpighian tubule (Skaer 1993) allows a more detailed examination of this enigmatic enzyme than would normally be possible and has provided novel insights into the tubule itself. First, the expression pattern delineated by the reporter genes in c507, c232, and \(l(3)07028\) defines a lower tubule domain (Sözen et al. 1997) that had not been detected by classical analysis (Wessing and Eichelberg 1978), but which we now know corresponds with the reabsorptive, rather than secretory, portion of the tubule (O'Donnell and Maddrell 1995). Second, reporter gene expression appears precisely to match the expression of Aph-4 mRNA (by in situ hybridization) and of alkaline phosphatase enzyme activity (by histochemistry) to a population of \(25 \pm 0.4\) cells. It is relatively rare for an enhancer-trap pattern to display such close agreement with the gene into which it inserts, and this is particularly interesting in a region where the gene density is so high, with two other genes within a kilobase, each with very different expression patterns. Third, the gene is probably essential because one of three insertions in the upstream sequence is semilethal. Fourth, the phenotypes of these insertions are unusual; rather than producing nulls or hypomorphs, they appear to interrupt a control region that specifies the lower tubule domain in some combinatorial fashion. Although expression of alkaline phosphatase in lower tubule is re-

**Figure 8.**—Effect of homozygosity for P element c507 on fluid secretion. Secretion rates were measured in Oregon-R (empty square) or c507/c507 homozygous flies. Tubules were challenged at 30 min with maximal concentrations of agonists known to act on principal or stellate cells. (A) \(10^{-5}\) m cardioactive peptide 2b (CAPb); (B) \(10^{-3}\) m cyclic AMP; (C) \(10^{-3}\) m Drosophila leucokinin. Data are presented as mean ± SEM, with the number of separate tubules in each experimental group shown in parentheses.
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Figure 9.—Nucleotide sequence of the l(3)96601 gene. Uppercase nucleotide sequences are from cDNA, lowercase from genomic DNA. The hypothetical 223-amino-acid translation product is indicated. The putative polyadenylation signal sequence is underlined. The 5' end of the transcriptionally convergent dbt gene (Kloss et al. 1998; B. Kloss, personal communication) and the 3' end of the transcriptionally convergent Aph-4 gene (pMY51 insert) are shown in italic. The cDNA sequence has been deposited in the EMBL/GenBank/DDBJ database and assigned accession no. X94917.

Reduced, it is greatly enhanced in the main segment. Our phosphatase, which is usually a membrane-associated enzyme, may disrupt this order and so nonspecifically best explanation of this at present is that the boundary between main and lower tubule domains is sharpened reduce the performance of the tissue, both of agonists that usually act on the principal cell (CAP2b and cAMP) by interacting positive and negative regulatory elements and that the P-element insertions separate these control and of those that act on the stellate cell (Drosophila leucokinin). The more distal, lower-tubule-specifying control region is thus What insights can we gain as to the role of alkaline phosphatase in tubule, or indeed in brain? In humans, moved 8 kb away from the more proximal, main segment control region and can no longer repress it. As there are multiple isozymes of ALP (McComb et al. 1979); the function of these is not well understood. There are a few kilobases between the CanA1 and Aph-4 coding regions (Figure 3), this might be a particularly However, ALP is a marker of osteoblast lineages, and hypophosphatasia, a recessive human disease with multi-rewarding area in which to perform a promoter analysis. How can the ectopic expression of alkaline phosphatase lead to a reduction in fluid secretion? In the fluid secretion assay, the lower tubule sits outside the bathing drop, and so dies; it acts merely as a conduit for the urine secreted by the main segment. The loss of alkaline phosphatase activity from the lower tubule is thus un-likely to influence measured rates of fluid secretion, evident, but the homozygous mutant mice died from seizures, apparently from an inability to metabolize vitamin B6 (Waymire et al. 1995). This probably reflects a role for ALPL/TNAP as an ectoenzyme, anchored to the cell surface, that converts pyridoxal-5'-phosphate to pyridoxal. The suggestion from the vertebrate literatureis that alkaline phosphatase can play a role in the metabolism of calcium phosphates. Interestingly, the
tubules are sites for deposits of calcium and magnesium phosphates, in the form of luminal concretion bodies, or spherites (Wessing and Eichberger 1978). These bodies have traditionally been considered to be sites of “storage excretion” of phosphates of calcium, magnesium, and other metals. As these spherites pack into the initial segments of the anterior tubules, it is possible that the deposits are laid down in the initial segment and then pass though the tubule to be excreted in the urine. Alkaline phosphatase on the apical surface of the lower tubule might allow for the selective reclamation of calcium when needed. This might explain the semithal nature of the mutations; the severity of disruption of calcium metabolism might depend on the nature of the diet.

Alkaline phosphatase activities in vertebrate brain are generally lower than those in liver and kidney (McCorm et al. 1979); however, in neuronal tissue, intense alkaline phosphatase activity has been located in neuronal cell bodies and processes in some parts of central and peripheral nervous systems of various animals such as mouse (Sood and Mulchandani 1977), rat (Nandy and Bourne 1963), guinea pig (Song et al. 1994), monkey (Friede 1966), and fish (Sood and Sinha 1983). At the ultrastructural level, the enzyme has been localized on the outer surface of plasma membranes of nerve cell bodies and dendrites (Miyahara et al. 1967; Mori and Nagano 1985) and postsynaptic membrane and synaptic vesicles (Sugimura and Mizutani 1979). Biochemical studies also suggest that alkaline phosphatase is associated with synaptic vesicles isolated from bovine cerebral cortex (Zisapel and Haklai 1980). In view of these facts that the ALP activity has been localized to discrete subsets of neurons in different species, it is suggested that the ALP has a function related to particular features of the reactive neurons. From the available evidence, it appears that the ALP may play some role in transmembrane transport (possibly of calcium) and cell differentiation or proliferation in the nervous tissue.

Although the full size of the ALP gene family in Drosophila is not yet known, we can hypothesize that the vertebrate neuronal and kidney roles, associated with expression of the liver/bone/kidney (tissue nonspecific) isozyme, are both played by transcripts of the analogous Drosophila gene, Aph-4. It may also be of interest that alkaline phosphatase (EC. 3.1.3.1) and protein phosphatase (also called calcineurin) (EC. 3.1.3.16) genes are located in the same chromosomal region at 100B. Another kind of phosphatase, acid phosphatase (EC. 3.1.3.2), is located at 99C5-7 of the same chromosome based on deletion mapping (Frisardi and MacIntyre 1984; Chung et al. 1996). All the above three phosphatases are classified as “phosphoric monoester hydrolases” (International Union of Biochemistry 1979). A similar clustering has been observed for human alkaline phosphate genes (Harris 1989). Possibly other phosphoric monoester hydrolase genes will be found near the 100B region.

We thank Greg Stewart, Ping Li, Elaine Cleary, Ji Luo, Douglas Armstrong, Shireen Davies, and Jonathan Sheps for their help with this work. We also thank Peter Deak for P{PacW} lines, Nicole Mozden and Allan Spradling for line (I)(3)07028, Brian Kloss for dbt, Claude Klee for CanA1. This work was supported by the U.K. Biotechnology and Biological Sciences Research Council and the Medical Research Council, and by the Wellcome Trust.

LITERATURE CITED


