Activity variation in acid phosphatase (ACPH) allozymes of *Drosophila virilis* resulting from two different *Acph* gene transcripts.

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Abstract

To reveal the mechanism underlying ACPH allozyme activity variation in *D. virilis*, the acid phosphatase allelic genes *Acph-1*, *Acph-2*, and *Acph-4* and their transcripts were isolated and characterized. Each gene includes six exons. Two major transcripts are formed by alternative splicing of exon 5, with two proteins of differing lengths produced as a consequence. Because exon 6 encodes a transmembrane segment, the long protein was inferred to be the membrane-bound form and the short protein the soluble one. Semi-quantitative and quantitative real-time PCR analyses of these transcripts revealed that the membrane-bound protein had similar expression levels among the three allelic genes. In contrast, the soluble protein showed variable expression: levels of *Acph-4*, *Acph-2*, and *Acph-1* were high, medium, and low, respectively, with their expression ratio (4.6:3.1:1) corresponding approximately to relative enzyme activities observed by gel electrophoresis. Comparative analysis of *Acph* nucleotide sequences of *D. virilis* and related species revealed that the activity differences are not due to variation in active sites, disulfide bonds, or glycosylation sites.

Introduction

Acid phosphatase (ACPH; EC 3.1.3.2), a lysosomal enzyme, was first described in the genus *Drosophila* by MacIntyre (1966). Immunological studies in *Drosophila* have shown that ACPH is a rapidly evolving protein (Collier *et al.*, 1990), and *Acph* has been extensively used as an allozymic marker in evolutionary studies of the genus (Ayala and Tracey, 1974; Kojima *et al.*, 1970; MacIntyre, 1966). Three allelic forms of ACPH specified by the *Acph* locus have been reported in the Japanese population of *D. virilis* (Ohba, 1977), with *Acph-2* comprising more than 98% of alleles. The three allelic enzymes (ACPH-1,-2, and -4) show activity differences based on the intensities of the allozyme bands (Narise, 1976). Five electrophoretic variants of ACPH have been found in a natural population of *D. subobscura* but exhibit no activity differences (Loukas *et al.*, 1979). On the basis of cellular localization experiments involving *D. virilis* ACPH, Narise (1985) has suggested that the allozymes are lysosomal enzymes, and that the variation in their activities is caused by differences in their lysosome-anchoring abilities.

Structures of two human ACPH proteins have been deduced from their gene structures. Leucocyte lysosomal acid phosphatase, composed of 11 exons, is a membrane-bound protein (Geier *et al.*, 1989), while prostatic acid phosphatase (Sharief and Li, 1992), which lacks exon 11 encoding the transmembrane segment, is a soluble protein. In *Drosophila*, the *Acph* gene was first cloned and sequenced in *D. melanogaster* (Chung *et al.*, 1996) and then in three species of the subobscura group (*D. subobscura*, *D. guanche*, and *D. madeirensis*) (Navarro-Sabate *et al.*, 1999). In these species, the *Acph* gene is organized into five exons interrupted by four short introns, with a single transcriptional start site and an additional intron present in the 5' leader region. I previously characterized the nucleotide sequence of the *Acph-2* allozyme gene of *D. virilis* and its transcripts (Kitagawa, 2003). The *Acph* gene was found to possess six exons, as the third exon present in other *Drosophila* species was divided into two exons. In addition, two transcriptional start sites in the 5' region and two spliced forms of exon 5 were identified. As a result of this variable splicing, the *Acph-2* gene encodes two major transcript forms having differing lengths. Navarro-Sabate *et al.* (1999) have reported that the nucleotide sequence surrounding the last exon encodes the transmembrane region of the enzyme protein. As described above, the ACPH of *D. virilis* is a lysosomal enzyme. The two types of transcripts may, therefore, play different roles, either penetrating or adhering to the lysosomal membrane. On the other hand,

Kim *et al*. (2014) have recently indicated that variable activities of apuric/apyrimidinic endonuclease 1 are caused by an amino acid substitution in this enzyme protein.

This study aimed to clarify whether differences in activities of *D. virilis Acph* allozymes were due to an amino acid replacement in active sites of the protein or were instead a consequence of different transcripts resulting from alternative splicing of the *Acph* gene. To accomplish the study objective, I analyzed *Acph* allozyme nucleotide sequences of *D. virilis* and related species and the deduced primary structure of ACPH allozyme proteins. I also investigated the expression pattern of the two transcripts arising from alternative splicing.

Materials and Methods

Drosophila *Samples*

Drosophila virilis and related species were used to survey ACPH protein allelic variation (Table 1). Lines of these species, collected mainly from Japanese populations, were maintained as isofemale lines and supplied by the laboratories listed in Table 1.

Table 1. Allozyme lines of *Drosophila virilis* and related species used in this study.

Species	Electrophoretic allele	Collection location and year	Name
D. virilis	Acph-1	OMAEZAKI, JAPAN, 1980	ViAc1-OZ[f]
	Acph-1	HORIOKA, JAPAN, 1999[c]	ViAc1-HO
	Acph-1	KYOTO, JAPAN, 2002[d]	ViAc1-KY
	Acph-2	OMAEZAKI, JAPAN, 1980[e]	ViAc2-OZ80[f]
	Acph-2	HORIOKA, JAPAN, 1983	ViAc2-HO
	Acph-2	OMAEZAKI, JAPAN, 1978	ViAc2-OZ78
	Acph-2	Mexico[a]	ViAc2-ME
	Acph-4	OMAEZAKI, JAPAN, 1980	ViAc4-OZ80[f]
	Acph-4	OMAEZAKI, JAPAN, 1988	ViAc4-OZ88
D. lummei	Acph-4	SAKATA, JAPAN[b]	Lum-sak
	Acph-4	KEMI, FINLAND[b]	Lum-kemi
D. novamexicana	Acph-3	Unknown[b]	Nov
D. ezoana	Acph-2	JAPAN[a]	Ezo

[a] Obtained from the National Drosophila Species Resource Center (stock numbers 15010-1051.48 [*D. virilis*] and 15010-1021.0 [*D. ezoana*]).

[b] Supplied by H. Watabe, Hokkaido University of Education.

[c] Supplied by K. Tsuno, Meikai University.

[d] Supplied by M. Ito, Kyoto Institute of Technology.

[e] Sequenced in a previous study (Kitagawa 2003).

[f] Previously raised in my laboratory and then used as *D. virilis* representative lines.

Detection of Acid Phosphatase by Gel Electrophoresis

Two days after eclosion, adult flies were homogenized in 10μL distilled water and centrifuged for 5 min at 14,400 ×*g*. The supernatant was then subjected to polyacrylamide gel electrophoresis on a 7.5% gel at pH 4.3. Phosphatase activity was localized by the diazo coupling method using 0.1 M acetate buffer (pH 5.0) containing 0.5 mg/mL disodium α-naphthyl phosphate and 0.5 mg/mL Azoic Diazo Component 48 (Narise, 1984).

DNA Sequencing

Preparation of genomic DNAs from adult flies and genomic library construction were performed as previously described (Tominaga *et al.*, 1992). *Acph* genes of three *D. virilis* lines (ViAc1-OZ, ViAc4-OZ80, and ViAc4-OZ88) were screened using *Acph-2* cDNA as a probe. Positive clones were subcloned using a pBluescript SK+ vector (Stratagene, USA). *Acph* genes of other lines of *D. virilis* and additional species were amplified by genomic PCR. VG-F1 and VG-R1 primers (Table 2) were used to amplify genomic DNA of *D. virilis*, *D. lummei*, and *D. novamexicana*, with VG-F1 and VG-R2 primers (Table 2) used for *D. ezoana*. PCR amplifications were performed in 50-µL volumes containing 25 µL PrimeSTAR HS reaction mix (Takara, Japan), 10 pmol of each primer, and 1 µL genomic DNA. PCR conditions consisted of a denaturation step of 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 55°C for 5 s, and 72°C for 2 min. The PCR products were cloned into a PCR-script vector (Stratagene) and sequenced using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) on an ABI PRISM 310 Genetic Analyzer.

Table 2. Oligonucleotide primers used in this study.

Name	Sequence (5' - 3')	Application[a]
VG-F1	GGCTCAGTCTCCCGTTTG	Genomic PCR
VG-R1	GCTCGAAGTCGCGAGCTGAAC	Genomic PCR
VG-R2	AAGGCCTATACTCGTAAATGC	Genomic PCR
VA-RT	P-GTAGATCATCAGGC	5'-RACE
VA-A1	ATAAATGACTTAATGGGCCGACCGCTA	5'-RACE
VA-A2	TCAAAGAGATCTTTGTAACGTGCCCA	5'-RACE
VA-S1	GAGAGCAAGCATAAGAAACAACTGTCG	5'-RACE
VA-S2	ATACAGCGCACATGATACCACAATTGC	5'-RACE
VA-F	CAACTGTCGCCGGATCGGT	3'-RACE
vT-F1	TGGTCACACTATCTTATCGAC	RT-PCR, qRT-PCR
vT-F2	GTGAGCAACGCGATTGTGCAT	RT-PCR
vT-R1	ATCAGAATGCCTGTTGCTGC	RT-PCR
vT-R2	GTACTTCCCCATCTTCGACA	qRT-PCR
nT-F1	TGGTCACACTATTTTATCGAC	RT-PCR
nT-F2	GTGAGCAACGCGTTTGTGCAT	RT-PCR

[a] RACE, rapid amplification of cDNA ends; RT-PCR, semi-quantitative reverse transcription PCR; qRT-PCR, quantitative real-time PCR

mRNA Isolation

Adult-fly mRNA was isolated using a QuickPrep mRNA Purification kit (GE Healthcare, UK) according to the manufacturer's protocols. After determination of concentration and purity on a UV photometer, mRNA was stored at −80°C.

Rapid Amplification of cDNA Ends (RACE) Analysis

For 5' RACE, 1 µg of mRNA was reverse-transcribed into cDNA using a 5'-Full RACE Core Set (Takara) in the presence of VA-RT primer (Table 2). The reverse transcription was performed under the following conditions: 30°C for 10 min, 50°C for 60 min, and 80°C for 2 min. The resulting cDNA was amplified in two rounds using an LA PCR kit (Takara), with VA-A1 and VA-S1 primers used for the first step followed by VA-A2 and VA-S2 primers in the second step (Table 2). PCR amplifications consisted of 25 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s. For 3' RACE, 1 µg of mRNA was reverse-transcribed into cDNA using a 3'-Full RACE Core Set (Takara) in the presence of oligo dT-3 site adaptor and

VA-F (Table 2) primers. PCR amplifications consisted of 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 2 min.

Semi-quantitative reverse transcription PCR (RT-PCR)

cDNAs were synthesized from 1 µg mRNA by RT-PCR using a PrimeScript RT-PCR kit (Takara) and oligo dT primer. The PCRs were performed with the following primer sets: vT-F1 and vT-R1 (for type A) and vT-F2 and vT-R1 (for type B) for *D. virilis* and *D lummei* and nT-F1 and vT-R1 (for type A) and nT-F2 and vT-R1 (for type B) for *D. novamexicana* and *D. ezoana* (Table 2). The resulting PCR products were separated on a 0.7 % agarose gel and visualized with a UV transilluminator.

Quantitative Real-time PCR (qRT-PCR)

Reverse transcription was performed at 37°C for 15 min in 20-µL volumes containing 10 µL of 5× PrimeScript RT master mix (Takara) and 1 µg mRNA. qRT-PCR was performed on a Thermal Cycler Dice Real Time System Lite (Takara). To verify the qRT-PCR results, the *Gapdh* gene was used as an internal control, with *Gapdh* gene-specific primers Gapdh-F (5'-TGGGCTATACCGATGAGGAG-3') and Gapdh-R (5'-CAAGTCAATGACACGGTTGG-3') designed to amplify a 160-bp fragment. The vT-F1 and vT-R2 primer sequences used to amplify *Acph* transcripts are shown in Table 2. qRT-PCRs were performed in 25-µL volumes containing 12.5 µL 2× SYBR Premix Ex *Taq* II (Takara), 10 µM of each primer, and 1 µL diluted cDNA. qRT-PCR amplification conditions consisted of a denaturation step of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

Data Analysis

Clustal W (Thompson *et al.*, 1994) was used to align the nucleotide sequences.

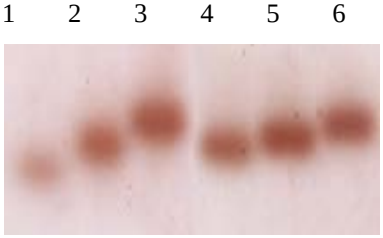


Figure 1. Results of an electrophoretic assay of adult fly extracts. Lanes 1–3 are from representative *Drosophila virilis* lines (1: ACPH-1, 2: ACPH-2, and 3: ACPH-4); lanes 4–6 correspond to related *Drosophila* species (4: Ezo, 5: Nov, and 6: Lum-sak). Details of the different lines are given in Table 1.

Results and Discussion

As previously reported by Narise (1976), the three allelic enzymes (ACPH-1, ACPH-2, and ACPH-4) of *D. virilis* from natural Japanese populations exhibit dramatically different activities (Figure 1, lanes 1–3 and Table 1). It was first necessary to clarify whether the differences in activity among ACPH allozymes are restricted to Japanese-specific populations or are more widely present in *D. virilis* and related species. Ten newly obtained strains of *D. virilis* and closely related species were, therefore, analyzed according to their electrophoretic mobility and using activity staining. On the basis of mobility and activity, ACPH-1 was identified in two *D. virilis* lines, with three lines characterized by the presence of ACPH-2 and one line possessing ACPH-4 (data not shown). Among related species, ACPHs of *D. ezoana* and *D. lummei* migrated at the same rate as ACPH-2 and ACPH-4 of *D. virilis*, respectively (Table 1). As shown in Figure 1 (lane 5), however, the mobility of the ACPH protein of *D. novamexicana* was halfway between that of ACPH-2 and ACPH-4. I designated this protein, which has not been previously reported, as ACPH-3. In regard to activity in *D. virilis*, the ACPH-4 enzyme showed the highest activity and ACPH-1 the least, with ACPH-2 intermediate (Figure 1, lanes 1–3). Among the three related species, in contrast, the ACPH-4 allozyme exhibited similar levels of activity in spite of differences in mobility (Figure 1, lanes 4–6). Differences in both activity and mobility of ACPH allelic enzymes may thus be specific to the *Acph* locus of *D. virilis*. Consequently, an analysis of *Acph* gene nucleotide sequences of eight lines of *D. virilis* and four lines of related species was performed.

Acph genes of eight *D. virilis* lines, two *D. lummei* lines, and one line each of *D. novamexicana* and *D. ezoana* were sequenced in this study (Table 1). A 4.2-Kb region including 0.6 Kb of the 5' region and 1.4 Kb of the 3' region of the *Acph* gene was sequenced in positive clones of three lines (ViAc1-OZ, ViAc4-OZ80, and ViAc4-OZ88); for the other lines, sequences of a 3.3-Kb PCR fragment including 0.6 Kb of the 5' region and 0.7 Kb of the 3' region were determined. The resulting *Acph* gene sequences were submitted to GenBank under the following accession numbers: AB271538 (ViAc1-OZ), AB986228 (ViAc1-HO), AB986229 (ViAc1-KY), AB986230 (ViAc2-HO), AB986231 (ViAc2-OZ78), AB986232 (ViAc2-ME), AB986233 (ViAc4-OZ80), AB986234 (ViAc4-OZ88), AB986235 (Lum-sak), AB986236 (Lum-kemi), AB986237 (Nov), and AB986238 (Ezo). The *Acph* genes of *D. virilis* and related species are organized into six exons. The nucleotide sequence around exon 1 encodes the signal peptide and exon 6 encodes the transmembrane region (Kitagawa, 2003). Nucleotide sequences of the 10 *Drosophila* lines were aligned with 3,479 bp of the ViAc2-OZ80 *Acph* gene, consisting of a 1,308-bp coding region and a 2,171-bp noncoding region. Only one site of nucleotide substitution, in the 3' region, was identified among the three *Acph-1* lines. Ten single-nucleotide substitutions and 3 insertions/deletions (indels) were found among the four *Acph-2* lines, with 23 substitutions and 6 indels, respectively, detected between the two *Acph-4* lines, and 26 substitutions and 3 indels between the two *D. lummei* lines. No nucleotide substitutions were found among the coding sequences of the three *Acph-1* lines and among the four *Acph-2* lines. Four synonymous substitutions were found between the two *Acph-4* lines and between the two *D. lummei* lines. On the other hand, no amino acid replacements were observed between lines of the same allelic form (data not shown).

The *Acph* gene encodes a precursor protein of 435 amino acids. Signal peptide (positions 1–29) and transmembrane domain (positions 384–435) regions are subsequently cleaved off to yield the mature protein. The mature protein was found to contain three noncontiguous residues (positions 93, 122, and 298) and two motifs (positions 52–58 and 97–100) for catalytic activity, five cysteine residues (positions 171, 322, 356, 360, and 380) for disulfide binding, and three motifs for glycosylation (positions 219–221, 229–231, and 343–345). All of these characteristics were found to be conserved across the *Drosophila* lines used in this experiment.

Table 3. Amino acid substitutions in deduced ACPH-1 and ACPH-4 sequences of *D. virilis* and related species relative to ACPH-2 of *D. virilis.*

	Position																						
	6	13	14	20	36	39	52	106	140	164	188	199	276	278	305	309	312	332	334	358	363	426
ACPH-2	H	F	F	H	G	A	F	A	N	V	Q	D	T	L	M	L	F	N	R	V	S	N
ACPH-1	.	.	.	.	.	.	.	.	.	.	**K**	.	.	.	.	.	.	.	.	.	.	.
ACPH-4	.	V	L	D	.	.	.	.	**D**	I	.	.	.	.	.	.	.	.	**S**	.	.	.
Lum	.	V	L	D	.	.	.	.	**D**	I	.	.	.	.	.	.	.	.	**S**	.	.	.
Nov	.	V	L	D	S	.	Y	.	**D**	I	.	.	.	.	.	I	.	.	.	.	.	.
Ezo	Y	V	L	D	**R**	P	.	S	**D**	I	.	E	N	F	T	.	L	S	.	I	A	K

ACPH-2, ACPH-1, and ACPH-4 refer to allelic forms in *D. virilis*; Lum, Nov, and Evo are *D. lummei*, *D. novamexicana*, and *D. ezoana*, respectively. Dots indicate amino acids that are identical to those of *D. virilis* ACPH-2. Substitutions causing a charge alteration in the mature protein (No. 30-383) are shown in bold. All deduced amino acid sequences of a given allelic form in *D. virilis* were identical to one another.

Table 3 displays amino acid differences between ACPH-1 and ACPH-4 of *D. virilis* and related species compared with ACPH-2 of *D. virilis*. Four nucleotide substitutions were found in *Acph-1*, one of which was a nonsynonymous substitution leading to a single amino acid replacement (Q → K). This mutation altered the electrophoretic mobility by changing a single charge. Relative to *Acph-2*, 25 and 27 substitutions were found in ViAc4-OZ80 and ViAc4-OZ88, respectively, of which six changes were nonsynonymous substitutions. Two replacements (N→ D and R → S) were responsible for anodic charge differences in ACPH electrophoretic mobility. In the other *Drosophila* species, a charge alteration accompanying the amino acid replacement occurred at two sites (N→ D, R → S) in *D. lummei*, at one site (N→ D) in *D. novamexicana*, and at two sites (G → R, N → D) in *D. ezoana*. These charge alterations were consistent with the observed differences in electrophoretic mobility. Nevertheless, the amino acid replacements did not occur

at the catalytic residues, glycosylation sites, or disulfide bonds. The amino acid changes thus do not seem to be the main cause of the activity difference.

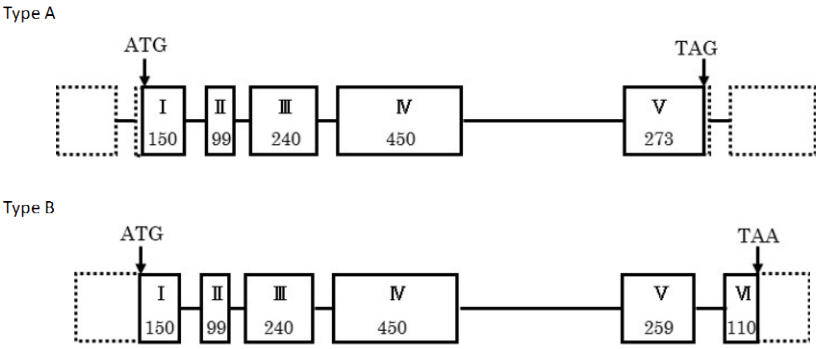


Figure 2. Alternative splicing patterns of *Acph* allozyme genes of *Drosophila virilis*. The dotted and open boxes indicate untranslated and coding regions, respectively. Roman and Arabic numerals in the open boxes are numbers of exons and nucleotides, respectively. Splicing for the type-A transcript begins at−296; the start site of the type-B transcript is at−194 in *Acph-1* and −167 in *Acph-4.*

RACE analysis was carried out using *D. virilis* lines representing *Acph-1* and *Acph-4* allelic forms (Table 1). As shown in Figure 2, two types of transcripts (types A and B) were found in both allelic forms. The coding region of the type-A transcript consisted of five exons, whereas that of type B comprised six exons. Exon 5 of type A was somewhat longer than that of type B and had a stop codon. Because the transmembrane domain, as previously mentioned, is found in exon 6, the type-A transcript yielded a soluble protein, whereas the type-B transcript encoded a membrane-bound form. RACE analyses of *Acph* in *D. lummei* and *D. novamexicana* confirmed the existence of two types of transcripts, similar to *D. virilis Acph*.

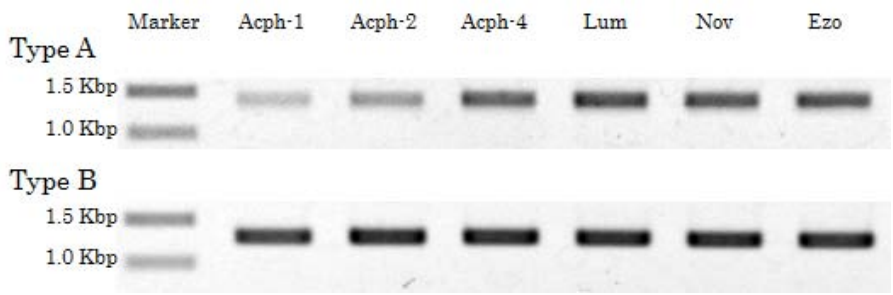
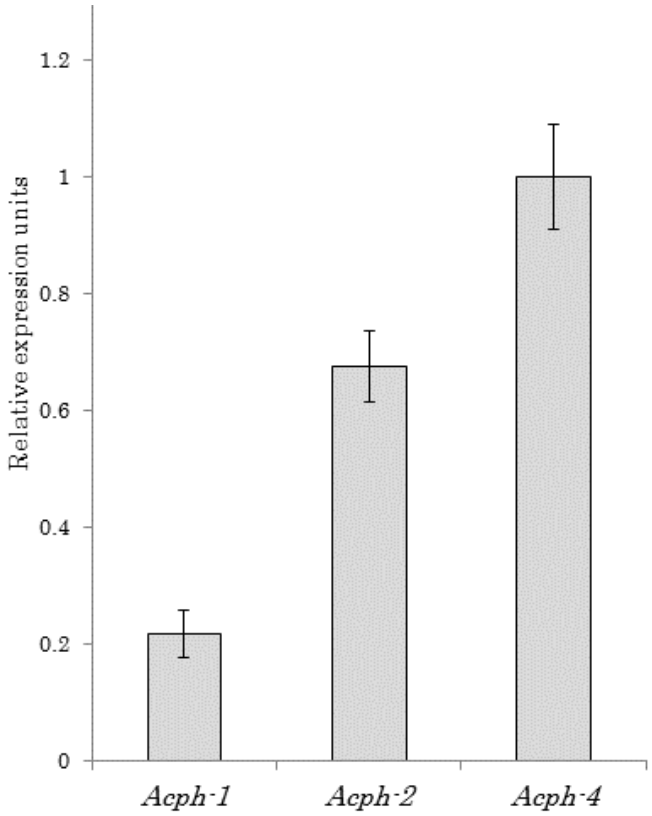


Figure 3. Results of expression analysis of *Acph* transcripts by semi-quantitative reverse transcription PCR. *Acph-1*, *Acph-2*, and *Acph-4* refer to representative *Drosophila virilis* lines harboring those alleles (see Table 1). Lum, Nov, and Ezo are *D. lummei*, *D. novamexicana* and *D. ezoana*, respectively.

Because two transcript types (A and B) were identified in the *Drosophila Acph* gene, their expression levels were examined by semi-quantitative RT-PCR. As revealed in Figure 3, expression levels of type-B transcripts were similar among lines. Type-A expression was variable among *Acph* allelic forms of *D. virilis*, while levels of *D. lummei*, *D. novamexicana*, and *D. ezoana* were similar to those of *D. virilis Acph-4*. Although the electrophoretic mobility of *D. ezoana* was identical to that of ACPH-2 (Figure 1), the expression level of the type-A transcript of *D. ezoana* was similar to that of *Acph-4*. This result indicates that the difference in the activities of *Acph* allozymes is restricted to *D. virilis*.

qRT-PCR was carried out to quantitatively compare type-A expression levels. Expression levels of *Acph-4* and *Acph-2* were, respectively, 4.6 and 3.1 times higher than those of *Acph-1* (Figure 4). These relative expression level differences are consistent with the observed ratio of electrophoretic band staining intensities. In other words, differences in expression levels of the type-A transcript encoding the soluble protein of ACPH were responsible for the activity differences in *Acph* allozyme bands.

ACPH is known to be a lysosomal enzyme in *Drosophila* (MacIntyre, 1966). On the basis of biochemical and morphological studies of developing *Drosophila* larvae, Jones and Bowen (1993) have reported that this enzyme is located in many organelles, with the soluble form appearing during the processes of morphogenesis or cellular degeneration. Narise (1985) has suggested that the variation in enzyme activity



observed on electropherograms is due to differences in the ability of the allozymes to be incorporated into lysosomes.

Figure 4. Results of quantitative real-time PCR analysis of type-A mRNA expression in *Acph* of *Drosophila virilis*. Representative *D. virilis Acph-1, Acph-2* and *Acph-4* lines were used (see Table 1). Expression was normalized relative to that of the *Gapdh* gene.

My study findings indicate that the *D. virilis Acph* gene encodes two types of transcripts—membrane-bound and soluble. The membrane-bound protein is likely located in organelles such as lysosomes, whereas the soluble form may be present in the cytosol of various tissues. The question arises as to why expression levels of transcripts encoding the soluble enzyme differ among *Acph* allozyme genes. One possibility is that this differential expression may be controlled by a regulatory system, such as a system involving enhancers and regulators. Thus far, however, I have been unable to identify any candidate nucleotide sequences in the regulatory region of this gene. Another unresolved question concerns tissue specificity of the different transcripts. In humans, as mentioned in the Introduction, leucocyte lysosomal acid phosphatase is a membrane-bound protein while prostatic acid phosphatase is a soluble protein (Sharief and Li, 1992). In addition, expression of lysosomal acid phosphatase in the testis and brain of mice is not uniform across tissues: high expression has been found to be restricted to spermatocytes in the testis and to neurons in the brain (Geier *et al.*, 1992). Expression of the type-A transcript of the *Acph* gene is thus most likely also differentially regulated in various organs and tissues. Further studies to investigate organ- and tissue-specific expression are required to clarify the mechanism responsible for the differing allozyme activities and to uncover the role of *Acph* in *Drosophila*.

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