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Amplification of the phosphorylation site – ATP-binding site cDNA fragment of the Na^+,K^+-ATPase and the Ca^{2+}-ATPase of *Drosophila melanogaster* by polymerase chain reaction

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In vitro DNA-amplification technique has been utilized to generate a 430 bp fragment of the Na^+,K^+-ATPase, and a 550 bp fragment of a Ca^{2+}-ATPase (the sarcoplasmic reticulum-type) of *Drosophila melanogaster* The oligonucleotide primers for the DNA-amplification (Polymerase Chain Reaction) had been designed on the basis of amino acid sequence motifs – the phosphorylation site and the ATP-binding site – conserved among members of the ATPase protein family Using the amplified cDNA-segments as probes, we demonstrated that there is one Na^+,K^+-ATPase and one Ca^{2+}-ATPase (sarcoplasmic reticulum-type) gene in the *Drosophila* genome Three different mRNA species are processed from the Na^+,K^+-ATPase gene and one from the Ca^{2+}-ATPase gene Developmental control in expression of the Ca^{2+}-ATPase gene was observed

Homology primer, Polymerase chain reaction, ATPase, Developmental control of gene expression, (*Drosophila melanogaster*)

1. INTRODUCTION

The ion-motive aspartyl-phosphate ATPases, catalyzing the active transport of various cations through biological membranes, form a protein family. They share common molecular architecture, basic mechanistic features of the ion transport and show amino acid sequence homology. In mammals the various ATPases appear in multiple forms (isoforms) encoded by closely related genes, e.g. 3 isoforms of the Na^+,K^+-ATPase [1]; two isoforms of the muscle cells sarcoplasmic reticulum Ca^{2+}-ATPase [2] and two isoforms of the plasma membrane Ca-ATPase [3] have been described.

For cloning new members of a protein family the cDNA or genomic libraries can be screened with a large-size DNA probe representing large portion or the full-size sequence of an established member of the family. Another approach is the application of short synthetic oligonucleotide probes for library screening designed on the basis of conserved amino acid sequence motifs [4]. Due to the degeneracy of the genetic code only relatively short and mixed oligonucleotides should be synthesized. The complexity of the mixed synthetic probe can be reduced by considering only the most preferred codon(s) of each amino acid. However, by reducing the complexity and the length of the probe its specificity will decline. It becomes difficult to establish the stringency of hybridization which discriminates between 'real and false' signal.

We utilized the recently introduced Polymerase Chain Reaction (PCR) [5] to rapidly clone new members of the ATPase gene-family, specifically the Na^+,K^+- and the Ca^{2+}-ATPase of *Drosophila melanogaster*.

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2. EXPERIMENTAL

2.1. *DNA and RNA source*

Genomic DNA and total RNA samples of *Drosophila melanogaster* as well as the cDNA-libraries (constructed in λgt10 vector) were gifts from Tom Kornberg, University of California at San Francisco

2 2 *Oligonucleotide primers*

Oligonucleotide primers were designed as described in section 3 and shown in fig.1. They were synthesized on an Applied Biosystem DNA Synthesizer, Model 300A and purified by polyacrylamide gel electrophoresis

2.3 *DNA amplification by polymerase chain reaction*

Purified DNA (0.3 μg) of various *Drosophila* cDNA-libraries was subjected to PCR amplification in the presence of 100 pmol of each PCR-primer of fig.1 following the procedure of Saiki et al [5]. Denaturing temperature of 92°C (1 min), annealing temperature of 55°C (2 min) and chain extension temperature of 72°C (2.5 min) was used 35 PCR cycles were performed and then the products were analyzed by agarose gel electrophoresis

2 4 *Sequencing of the PCR products*

The 430 bp PCR-product amplified from the *Drosophila* larvae library (fig.2, lane 1) and the 550 bp PCR-product of adult male and

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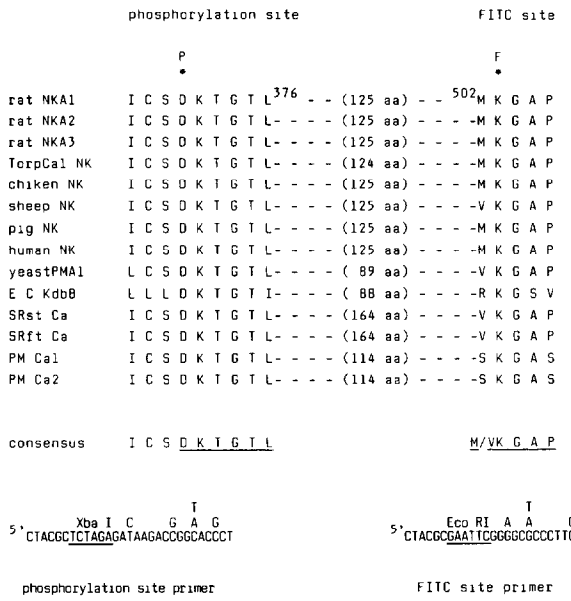

Fig 1 Design of the PCR-primers Members of the ATPase family included into the search are the following rat NKA1–3, three isoforms of rat brain Na,K-ATPase α-subunit [1], TorpCal NK, α-subunit of the *Torpedo californica* electroplax Na,K-ATPase [13], chicken NK, α-subunit of the chicken kidney Na,K-ATPase [14]; sheep NK, α-subunit of the sheep kidney Na,K-ATPase [15], pig NK, α-subunit of the pig kidney Na,K-ATPase [16], human NK, α-subunit of HeLa cell Na,K-ATPase [17], yeast PMA1, plasma membrane ATPase of yeast [18], E C KdbB, K-transport ATPase of *E coli* [19], SRst Ca and SRft Ca, sarcoplasmic reticulum Ca-ATPases of slow twitch and of fast twitch rabbit muscle cells, respectively [2], PM Ca1 and PM Ca2, two isoforms of the rat brain plasma membrane Ca-ATPase [3] The rat Na,K-ATPase α1-subunit numbering is used * and P indicate the Asp residue phosphorylated, * and F the Lys residue covalently labelled by fluorescent isothiocyanate (FITC), respectively Segments of the consensus sequences considered for primer design are underlined

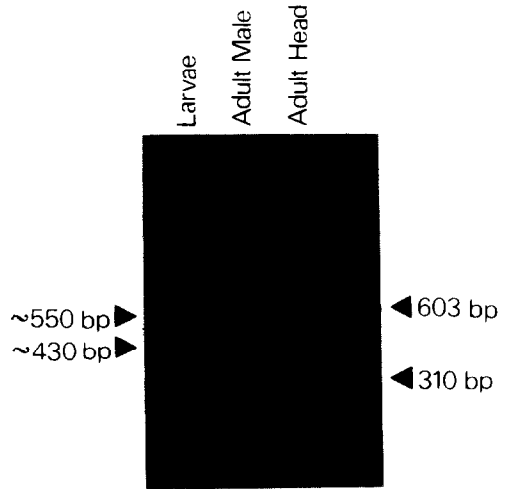

Fig 2 Amplification of cDNA segments from different *Drosophila* cDNA-libraries by PCR. Amplification from λgt10 cDNA-libraries constructed from larvae (instar I & II) developing stage (lane 1), from adult males (lane 2) and from adult heads (lane 3), respectively

adult head library (fig 2, lanes 2 and 3) were excised from the agarose gel, electroeluted and cloned into the *Sma*I site of the M13 sequencing vectors mp 18 and 19 by blunt end ligation Aliquots of the purified PCR-products were also digested with restriction enzymes *Eco*RI and *Xba*I and cloned into the *Eco*RI and the *Xba*I sites of the same sequencing vectors Sequencing was performed by the chain-termination method of Sanger et al [6]

2 5 *Southern and Northern hybridization*

Genomic DNA was digested with restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII The digestion products were fractionated on a 0 8% agarose gel, transferred to nitrocellulose paper and probed High stringency wash (65°C) has been applied.

Total RNA was isolated from embryos, larvae, pupae and adults The RNAs were size fractionated on a 1% agarose-formaldehyde gel, transferred to nitrocellulose paper and probed High stringency wash (65°C) has been applied

3. RESULTS AND DISCUSSION

The primers used for in vitro DNA-amplification were designed on the basis of sequence information deduced from members of the ATPase protein family. The essence of this approach is a search for conserved motifs in the amino acid sequences of proteins belonging to the same protein family to which the gene(s) to be cloned also belong. Several ion-motive ATPase sequences with a wide variety of species were included in the search (fig.1). Two regions of the sequences were found to show remarkable sequence similarity. The first region is located around the phosphorylation site (Asp-371, rat Na,K-ATPase α1 isoform numbering), where a 9 amino acid segment of the sequence (368–376) is conserved in all the known eukaryotic enzymes. The second region harbours the residue (Lys-503) covalently modified by fluorescent isothiocyanate (FITC). This region may be involved in ATP-binding [7]. Plasma membrane ATPases vary from the consensus sequence with Met or Val in position 502. Mixed oligonucleotide primers were designed on the basis of the consensus sequences around the phosphorylation site and the FITC-site (fig.1). To reduce the degree of degeneracy of the mixed oligonucleotides the codon usage bias characteristic for the *Drosophila* genes [8] was employed. Restriction endonuclease linker sequences were attached to the 5′ ends of the primer regions to facilitate directional cloning of the amplified products.

Various *Drosophila* cDNA-libraries were subjected to PCR amplification. Two main products were obtained as is shown in fig.2. After excision from the agarose gel the two *Drosophila* PCR-products (430 bp and 550 bp) were cloned into M13 sequencing vectors mp 18 and mp 19 and sequenced (figs 3 and 4).

Forty-two M13 clones with the 430 bp DNA segment had been sequenced. All the clones appeared to carry PCR-amplification products since they possessed the primer sequences. 29 of the 42 clones were found to carry identical sequence between the primer regions. This 375 bp-long segment (DrosNKPF) is identical with

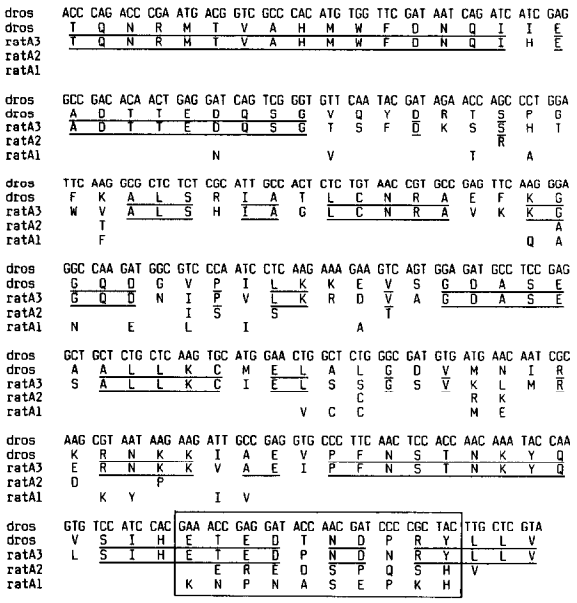

Fig.3 Nucleotide and deduced amino acid sequences of the phosphorylation site-FITC site segment of *Drosophila* Na,K-ATPase (DrosNKPF) The sequence of the PCR product amplified from the *Drosophila* larvae library starts after the phosphorylation site (at Thr-377 according to the rat Na,K-ATPase α1 subunit numbering) and ends before the FITC-site primer (at Val-501 by the same numbering) For comparison the amino acid sequences of the same regions of the three rat Na,K-ATPase α-subunit isoforms [1] are also shown Identical residues of the *Drosophila* sequence and those of the rat α3 sequence are underlined For the rat α2 and α1 sequences only the residues different from those in the α3-isoform are indicated The 489–498 region where the 3 rat isoform sequences are the most diverged is boxed

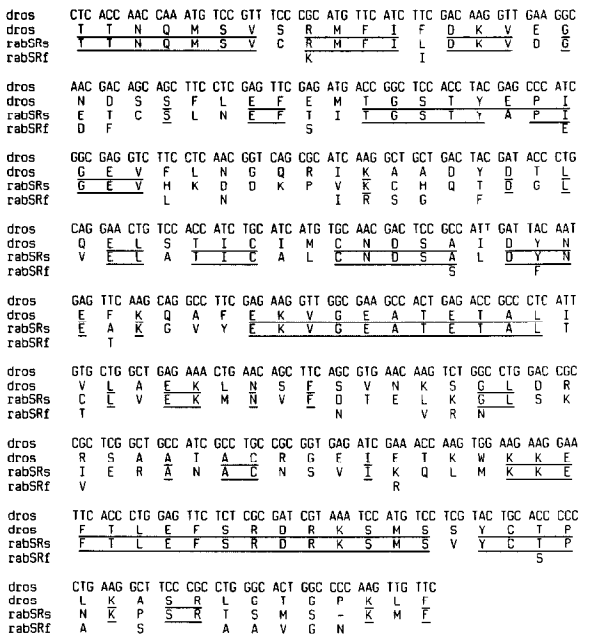

Fig 4 Nucleotide and deduced amino acid sequences of the phosphorylation site-FITC site segment of *Drosophila* sarcoplasmic reticulum-type Ca-ATPase The sequence of the 550 bp PCR product amplified from the *Drosophila* adult male and from the adult head library starts after the phosphorylation site primer (at Leu-355 according to the numbering of the sarcoplasmic reticulum Ca-ATPases of fast twitch rabbit muscle cells) and ends at the FITC-site primer (Phe-512 by the same numbering) For comparison the amino acid sequences of the same regions of the rabbit slow twitch and fast twitch muscle Ca-ATPase [2] are also shown Identical residues of the *Drosophila* sequence and those of the rabbit fast twitch form are underlined For the slow twitch form sequence only the residues different from those of the fast twitch form are indicated

the phosphorylation site-FITC site portion of the *Drosophila* Na,K-ATPase cDNA sequenced recently [9]. The published sequence and the sequence of the P-F region of fig.3 differ in two positions, both silent mutations affecting third codon bases. These variations could be due to strain differences in the Na,K-ATPase gene. The amino acid sequence between the phosphorylation site and the FITC site is the most variable among the 3 isoforms of the rat Na,K-ATPase [1]. From the sequence comparison of fig.3, it can be concluded that the DrosNKPF sequence is more closely related to the P-F region of the rat α3 isoform than to the other two isoforms. This is most obvious if one compares the 489–498 region (rat α1 isoform numbering) where the 3 rat sequences are rather diverged while DrosNKPF sequence matches at 8 of the 10 positions with the rat α3 isoform sequence (boxed on fig.3). The conservation of the 489–498 region is most probably due to functional reasons.

The nucleotide sequences of 13 clones could be aligned to various regions of the λ-phage sequence (not shown); consequently they are amplification products of the vector in which the libraries had been constructed.

The sequence of the 550 bp product had also been established. Six clones were sequenced; 3 of them showed identical sequence (fig.4). On the basis of amino acid sequence homology this sequence could be aligned to the P-F region of the rabbit sarcoplasmic reticulum-type Ca^{2+}-ATPase sequences. The percent values of identical sequence positions are 62% of the nucleotide and 59% of the amino acid sequence for the rabbit fast twitch muscle cell form; 63% and 60% for the slow twitch muscle cell form, respectively. These high values of identical sequence positions allow us to conclude that the amplified product is the P-F region of a sarcoplasmic reticulum-type Ca^{2+}-ATPase cDNA of *Drosophila melanogaster* (DrosSRCaPF).

The amplified DrosNKPF was used to localize the corresponding gene on the chromosomes. Using ^{35}S-labelled DrosNaKPF-probe, one Na^+,K^+-ATPase gene had been detected by in situ hybridization and mapped on the right arm of chromosome 3, in section 93A or B (not shown) which is in agreement with the results of Lebovitz et al. [9]. The existence of a single Na^+,K^+-ATPase gene in the *Drosophila* genome is further supported by Southern hybridization experiments of *Eco*RI-, *Hin*dIII- and *Bam*HI-digested genomic

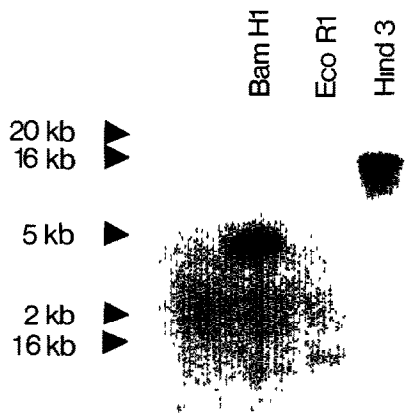

Fig 5 Southern hybridization of *Drosophila* genomic DNA digested with different restriction endonucleases DrosSRCaPF was used as probe In each lane 5 μg of digested DNA was applied

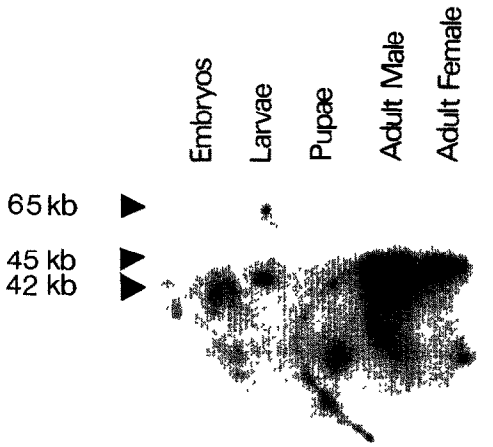

Fig 6 Analysis of mRNA abundancy of *Drosophila* Ca-ATPase in different developmental stages Northern hybridizations of total RNA obtained from different developmental stages with radiolabelled DrosSRCaPF In each lane 5 μg of RNA was applied

Drosophila DNA with the DrosNKPF-probe (not shown). Southern hybridization of the same digests with the DrosSRCaPF-probe suggests that the SR-type Ca^{2+}-ATPase is encoded by one gene in the *Drosophila* genome (fig.5; note that there is an *Eco*RI site in DrosSRCaPF). It is noteworthy that in mammals both enzymes are encoded by isogenes [1,2].

We analyzed the mRNA abundance of *Drosophila* Na,K-ATPase and Ca-ATPase in different developmental stages by Northern hybridization. Three mRNA-species with different sizes (4.2 kb, 4.5 kb and 6.5 kb) have been detected with the DrosNKPF probe in embryonic and in adult stage (not shown). This result is in harmony with those of Lebovitz et al. [9].

Developmental control of the mRNA abundance of *Drosophila* SR-type Ca^{2+}-ATPase was observed by Northern analysis using the DrosSRCaPF amplification product as probe (fig.6). No mRNA was detected in embryonic, larval or pupal stage; one mRNA-species with a size of 4.5 kb was present in the samples obtained from adult male and female. These findings are consistent with the PCR-amplification experiments of fig.2 where no 550 bp product (DrosSRCaPF) was detected from the amplification of the larval cDNA-library. This amplification product was only generated from the adult male and from the adult head library.

The cloning strategy described here yielded corresponding segments (the P-F region) of two ATPase-cDNAs from one single gene amplification experiment (fig.2). The common feature of these cDNAs – as predicted from the amino acid sequence data of the other members of the ATPase gene family – is the conservation of two functionally important motifs: the phosphorylation site and a site involved in ATP-binding (FITC-site). This simple prediction has been utilized for designing primers for the Polymerase Chain Reaction. Similar strategy was described recently by various reports [10–12].

The plasma membrane type Ca-ATPases clearly belong to the same ATPase family although their amino acid sequence around the FITC-site is somewhat different (fig.1). Since one of the deviations from the consensus sequence utilized for primer design affects the 3′ end of the FITC-site primer (Ser in the Ca-ATPase sequences, Met or Val in the consensus sequence) no chain extension is possible from this primer. This may be a plausible explanation why no amplification of cDNA-segments of this type of Ca-ATPases had been achieved from the *Drosophila* libraries studied.

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