University of South Bohemia

Faculty of Science

Department of Molecular Biology

BACHELOR THESIS

Developmental profile of putative ecto-5´-nucleotidase genes of *Drosophila melanogaster*

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Annotation:

In this work, expression of the genes predicted as ecto-5'-nucleotidases was monitored in different developmental stages of *Drosophila melanogaster*.

Anotace:

Tato práce se zabývá zkoumáním exprese genů s potenciální ektonukleotidázovou aktivitou v různých vývojových stádiích *Drosophila melanogaster*.

Annotation:

Diese Arbeit untersucht die Expression von Genen mit potenzieller Ektonukleotidase Aktivität in verschiedenen Entwicklungsstadien von *Drosophila melanogaster*.

I hereby declare under oath that the submitted bachelor thesis has been written solely by me without any third-party assistance. Additional sources or aids are fully documented in this paper, and sources for literal or paraphrased quotes are accurately credited.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my dissertation thesis, in full / in shortened form resulting from deletion of indicated parts to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

České Budějovice, 24 May 2010 .....

Radka Hobizalová

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CONTENTS

ABSTRACT

Extracellular adenosine plays an important role in organism, having an impact on immune, nervous and circulatory system. One way of the elevation of adenosine concentration outside the cell is the hydrolysis of ATP to ADP and AMP and final dephosphorylation of AMP to adenosine. The terminal step is catalyzed by ecto-5´-nucleotidase. The main function of the enzyme has been already investigated in mammalian systems including human. The question to be answered in our experiment was if it works in the same way in fruit fly. Four homologues of human ecto-5´-nucleotidase gene were found in fly genome database (FlyBase) and their expression in different developmental stages of Drosophila melanogaster was monitored by means of reverse-transcription polymerase chain reaction (RT-PCR). Six transcripts of three genes were shown to be transcribed in different developmental stages. With this knowledge, the material was prepared for further analysis of corresponding protein activity.

1 INTRODUCTION

1.1 ADENOSINE

Adenosine (Ado) is a naturally occurring purine nucleoside consisting of molecule of ribose sugar and adenine base connected by β-N_9-glycosidic bond. It can be found inside the cell (intracellular adenosine) as well as outside (extracellular adenosine). It serves multiple functions, namely it is a structural component of nucleic acids, an important energy storage molecule in the form of ATP, ADP and AMP, and also a signalling molecule.

1.1.1 Signalling via adenosine receptors

Signal transduction proceeds via activation of adenosine receptors by extracellular adenosine (Latini and Pedata, 2001). Adenosine receptors are seven-transmembrane-spanning G-protein coupled receptors that can be divided to 4 subtypes - A_1, A_{2A}, A_{2B} and A_3 (Fredholm, et al., 2001) Receptors are activated by physiological levels of adenosine except for A_{2B} receptor, which requires pathophysiological conditions with elevated adenosine concentrations (Fredholm, 2007). Adenosine is a preferred ligand at the receptors, although activation with inosine is also possible at A_3. The A_2 receptors preferably interact with stimulatory G_s proteins and the A_1 and A_3 receptors with inhibitory G_i/G_0 proteins (Fredholm, et al., 2001). Additionally, A_{2B} and A_3 receptors can also couple to Gq proteins, stimulating phospholipase activity (Feoktisov and Biaggioni, 1997). The signal usually takes pathway based on stimulation or inhibition of adenylyl cyclase and subsequent increase, respectively decrease of intracellular cyclic AMP level. Nevertheless, receptor activation can also be linked to other pathways (Haskó, et al., 2008).

1.1.2 Effects of extracellular adenosine

Extracellular adenosine affects many cells and organs via adenosine receptors. Its main function is to protect tissues and facilitate their regeneration if damaged. The accumulation of adenosine in the extracellular space occurs in response to various stress conditions. These include hypoxia, ischemia, inflammation or trauma (Linden, 2005; Fredholm, 2007).

Regulation of oxygen consumption

Firstly, adenosine plays a role in regulating oxygen consumption, more precisely it helps to increase oxygen supply/demand ratio. During hypoxia, oxygen supply deprivation, adenosine formation is stimulated which leads to vasodilatation. This results in increase of coronary blood flow and thus higher oxygen delivery to the hypoxic organ. This process is mediated by A_{2A} and A_{2B} receptors (Linden, 2005). On the other hand, activation of A_1 receptors helps to reduce oxygen demand in excitable tissues (heart, neurons) by promoting the neuronal conductance to potassium ions or inhibition of conductance to calcium ions (Dolphin, et al., 1986). The heart rate and neuronal activity are decreased.

Sleep-promoting factor

Inhibition of neuronal activity through action of A_1 receptor is also connected to sleep-promoting properties of adenosine. Extracellular adenosine accumulates in brain (basal forebrain area) during prolonged wakefulness. It causes a decrease in the activity of cholinergic cells which in turn induces sleep (Porkka-Heiskanen, et al., 2002). The major antagonist of adenosine, caffeine, is on the other hand believed to delay fatigue by binding to adenosine receptors instead of adenosine. The function of adenosine as neurotransmitter release inhibitor is blocked, resulting in increased neuronal activity (Fredholm, et al., 1999).

Ischemic preconditioning

Tissue protection is also provided by the ischemic preconditioning (ICP), with adenosine as one of the possible mediator (Fryer, et al., 2002). ICP means that short prior ischemic insult can prevent the tissue from subsequent prolonged ischemia. Adenosine produced in ischemic tissue (usually heart) contribute to the phenomena by activating A_1 and A_3 receptors, further transmitting the signal to kinase cascades and K_{ATP} channels. (Miura, et al., 2000).

Immunomodulation

By studying adenosine receptors agonists and antagonists, the evidence was obtained that adenosine participate in modulation of inflammatory processes via all adenosine receptors (Cronstein, 1999). Functions of various kinds of cells involved in machinery of immune response, such as neutrophils, monocytes or macrophages, lymphocytes, dendritic and mast cells, are regulated (Fredholm, 2007; Haskó, et al., 2008). Particularly A_{2A} receptor was found

to produce cellular effects inhibiting inflammatory actions. It prevents the production of cytokines, chemokines and oxygen radicals, prevent endothelial cell activation, and reduce significantly microvascular occlusion (Linden, 2005). One exception is A_{2B} receptor which showed rather pro-inflammatory behaviour in cells of some species (Zhong, et al., 2004; Ryzhov, et al., 2008).

Stimulation of angiogenesis

Stimulation of angiogenesis, the growth of new blood vessels, is a necessary part of tissue repairing mechanism after an injury. Adenosine serves as such stimulator by means of adenosine receptors. Activation of receptor A_{2B} or A_3 induces the release angiogenic factors (Feoktistov, et al., 2002; Feoktistov, et al., 2003) and A_{2A} receptors block the release of the anti-angiogenic protein thrombospondin 1 (Desai, et al., 2005), both promoting the vascular tube formation.

1.1.3 Adenosine metabolism

The availability of adenosine depends on its production, degradation, release and cellular uptake. Transport across the membrane is mediated by facilitated nucleoside transporters. Adenosine can be also transferred from the cell in form of ATP. Both intra- and extracellular ATP undergoes series of dephosporylation reaction leading to production of adenosine. Intracellularly, the conversion AMP→Ado occurs with help of cyto-5´-nucleotidase while the reverse reaction is catalyzed by adenosine kinase. Extracellular process of adenosine formation is catalyzed by series of ectoenzymes, including nucleoside triphosphate dephosphorylases (NTPDase) and ecto-5´-nucleotidases. Generated adenosine either interact with one of the adenosine receptors or undergo degradation to inosine via adenosine deaminase (ADA) and further transformation to uric acid (Blackburn, 2003; Haskó & Cronstein, 2004). In healthy tissues, levels of extracellular adenosine are maintained low as a result of the rapid uptake and metabolism. The physiological adenosine concentrations move around 0,01 to 1 μM (Haskó, et al., 2008). The adenosine cycle is depicted in Figure 1.

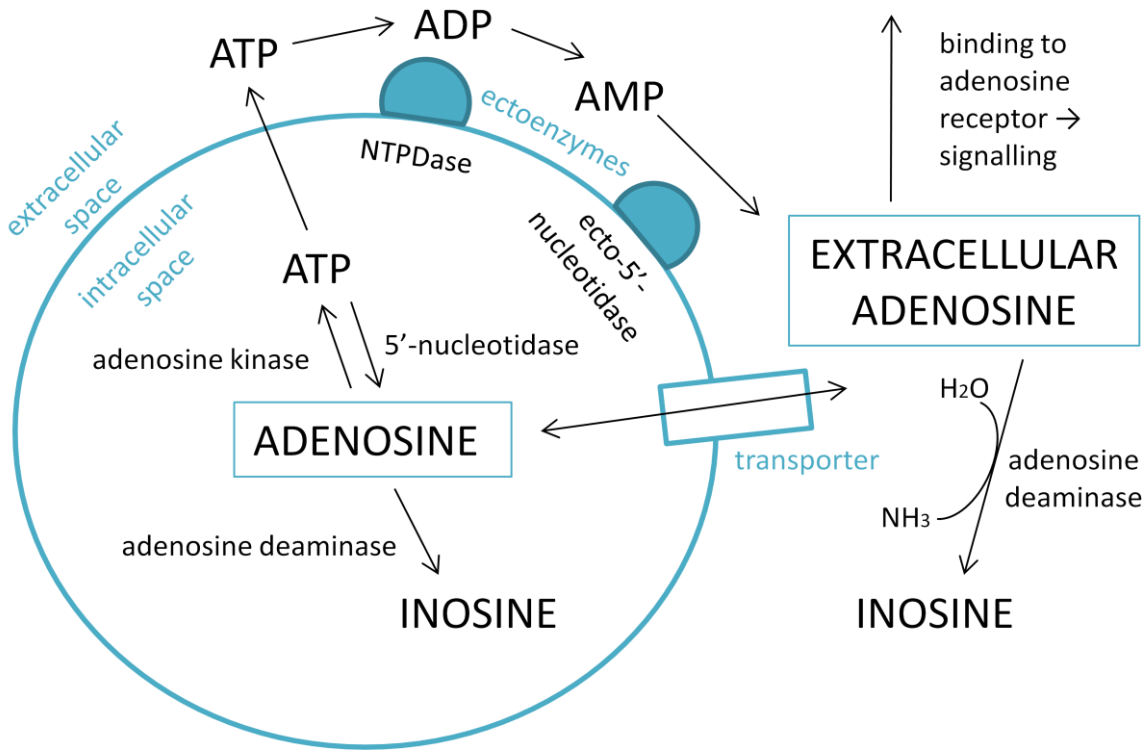

Figure 1: Adenosine metabolism pathways – release, uptake, production and degradation.

1.2 ECTO-5'-NUCLEOTIDASE

1.2.1 General description

Ecto-5'-nucleotidase is an enzyme consisting of two identical 60-80 kD subunits bound to the external face of the plasma membrane by a glycosyl phosphatidyl inositol linkage (GPI anchor). It belongs to the family of 5'-nucleotidases, being the only surface located representative. Other enzymes in this group are present in cytosol in soluble form (Zimmermann, 1992). According to HomoloGene database, presence of ecto-5'-nucleotidase gene was detected in number of vertebrates (e.g. human, mouse, rat, wolf, zebrafish) as well as in few insect species (*Drosophila, Anopheles*). In human and some other mammals, ecto-5'-nucleotidase is denoted as CD73.

1.2.2 Function

Primary function of ecto-5'-nucleotidase is the hydrolysis of extracellular nucleoside 5' monophosphates. 5'-AMP is a preferred substrate, yielding adenosine and phosphate as products (Zimmermann, 1992). Apart from that function, ecto-5'-nucleotidase is involved in

co-stimulatory signalling in T cells, lymphocyte adhesion to endothelium and controlling particular cell-cell interactions in immune system. It is also used as a marker of T and B lymphocyte differentiation. Low ecto-5'-nucleotidase activity can be observed on lymphocytes of patients suffering from a variety of immunodeficiency diseases based on block in lymphocyte maturation (Marinello, et al., 2000).

1.3 ADENOSINE IN *DROSOPHILA* MODEL

Extracellular adenosine effects have already been studied in *Drosophila melanogaster*, although to less extent in comparison to mammalian model.

1.3.1 Adenosine deaminase role

Recent publications deals mainly with the role of adenosine deaminase (ADA) in extracellular adenosine metabolism (Zurovec, et al., 2002; Dolezal, et al., 2005). The ecto- form of ADA, the enzyme catalyzing irreversible deamination of adenosine to inosine, has been proposed to regulate extracellular adenosine levels (Richard, et al., 2002). It was proven in mice that a lack of the enzyme causes uncontrollable elevation of adenosine/deoxyadenosine in tissues, resulting in pathological changes in organ systems and combined-immunodeficiency (Blackburn, et al., 1998). Investigation of ADA deficiency in *Drosophila* has given similar conclusions. Adenosine deaminase-related growth factor A (ADGF-A), gene showing ADA activity in *Drosophila* larvae, was used for the experiments based on homologous recombination mutagenesis. It was found that *adgf-a* mutation dramatically increases levels of adenosine and deoxyadenosine in the larval hemolymph and leads to larval death associated with fat body disintegration and the melanotic tumors formation (Dolezal, et al., 2003; Dolezal, et al., 2005). The a*dgf-a* mutants can be rescued by mutation in adenosine receptor (*adoR*), i.e. suppression of adenosine signalling. That indicates the great impact of signalling effects of extracellular adenosine on the phenotype (Dolezal, et al., 2005).

1.3.2 Ecto-5'-nucleotidase role

This work focuses on extracellular adenosine production in *Drosophila*, the role of potential ecto-5'-nucleotidases respectively. In the above section the mechanism of this process was described in general. Assuming that extracellular adenosine in *Drosophila* follow the same pathways, an equivalent of human or mammalian ecto-5'-nucleotidase should also be present in the flies and catalyze adenosine formation. In the *Drosophila* genome database, there were found four homologues to human gene coding for this enzyme - CG4827, CG30104, CG42249 and CG11883. For simplicity, they were abbreviated NT1, NT2, NT3 and NT4 in our work. NT3 was predicted to have two and NT4 three alternative transcripts. The scheme of the genes with all relevant data, including position of the gene on a chromosome, length of the gene sequences and mRNA transcripts or designed primers, is depicted in Figure 2 and summarized in Table 1.

Table 1: Investigated genes and the data related to them

gene	location	transcript	length (genomic)	length (mRNA)	forward primer	reverse primer
CG4827	2R	NT1	2120	1806	NT1-NOT-F	NT1-BST-R
CG30104	2R	NT2	2129	1828	NT2-ECO-F	NT2-NOT-R
CG42249	X	NT3B	3565	1699	NT3-ECO-F	NT3B-BST-R
		NT3C	4645	1719	NT3-ECO-F	NT3C-BST-R
CG11883	2R	NT4A	3229	2699	NT4AB-XHO-F	NT4-BST-R
		NT4B	2622	2173	NT4AB-XHO-F	NT4BC-BST-R
		NT4C	3071	2395	NT4C-XHO-F	NT4BC-BST-R

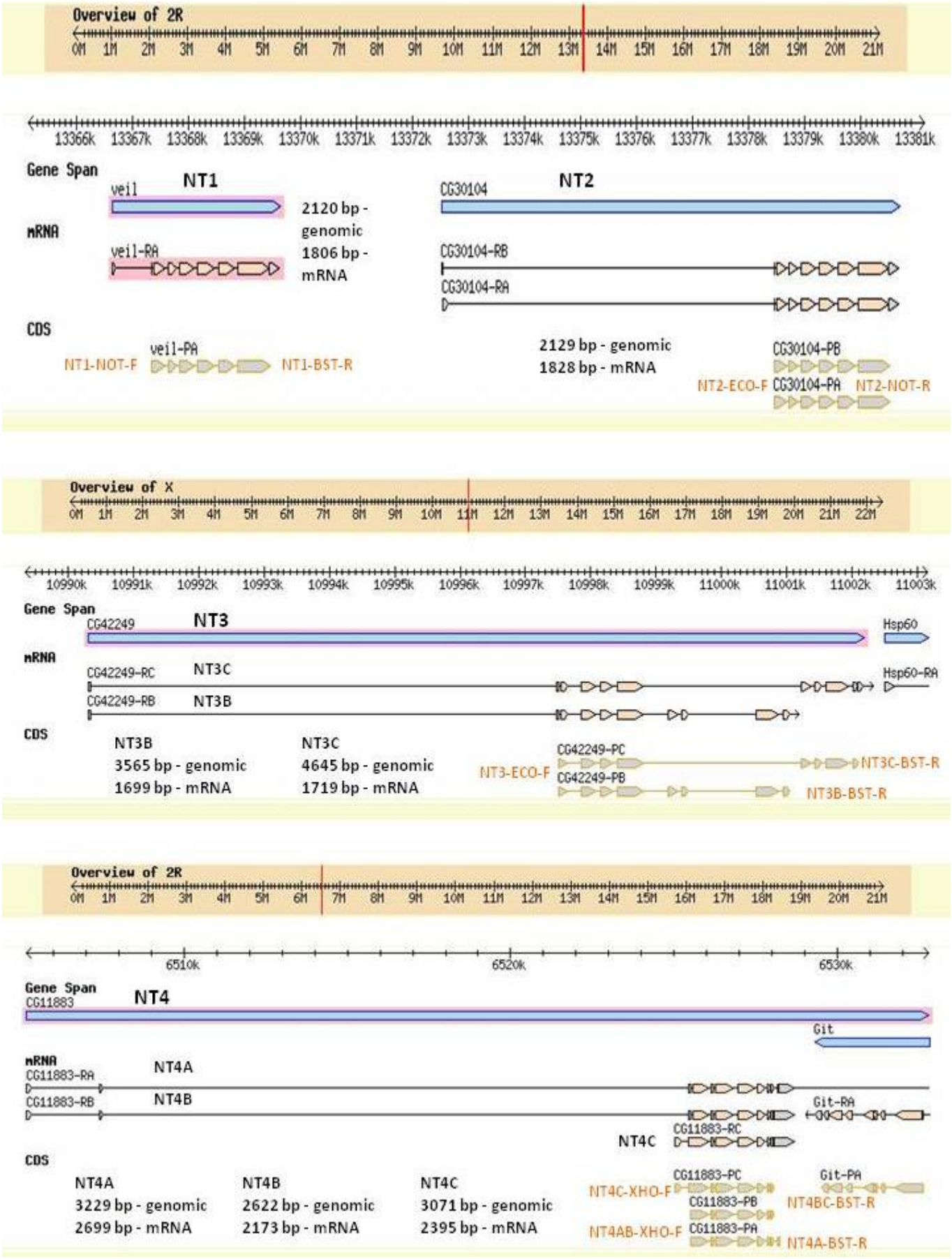

Figure 2: Scheme of genes with predicted 5´-nucleotidase activity (NT1 to NT4) according to FlyBase. Additional information: genomic and mRNA sequence lengths, designed primers (in orange).

2 AIM OF THE THESIS

The main aim of the thesis was to find out if any of the four genes is expressed in different developmental stages of fruitfly. That comprised several steps. At first, the annealing temperature of primers had to be optimized by performing polymerase chain reaction. In the following step, cDNA of 10 developmental stages of *Drosophila* was to be prepared and amplified by using the reverse-transcription and subsequent polymerase chain reaction. At last, the proof-reading polymerase chain reaction and purification procedure needed to be carried out for selected samples to prepare them for futher experiments.

3 MATERIALS AND METHODS

3.1 OPTIMIZATION OF PRIMERS

For the investigated genes, eleven primers were designed, with the length from 29 to 36 nucleotides (see Table 1). They contained the restriction sites (in green) for the cloning of cDNA sequences (discussed in Conclusion section). According to a table based on sequence length and number of C and G nucleotides, the annealing temperature of each primer was estimated. For each alternative transcript, three temperatures around the lower value of specific pair of primers (forward and reverse) were chosen for the polymerase chain reaction (PCR). PCR products were then visualized by agarose gel electrophoresis. The temperature showing the best results was used for next experiments.

Table 2: List of designed primers

gene	primer	sequence	length
NT1	NT1-NOT-F	CATCGCGGCCGCAAAATGTTACCTGCCCGTGGTC	34
	NT1-BST-R	GCTGTTCGAACCACTAGATGAATCTAGTTAGCAGGG	36
NT2	NT2-ECO-F	CAGAATTCGAAGATGCAGTCACGTTGGCTTTG	32
	NT2-NOT-R	AGATGCGGCCGCATCGCATTATCGACGCATCAAC	34
NT3	NT3-ECO-F	GTGAATTCGCAGATTCTGGTGCGTTGGAG	29
	NT3B-BST-R	GTATTTCGAATCAGTTGAGCACTGTGATGCG	31
	NT3C-BST-R	CTACTTCGAACGACCATACTATAAAGTCTAAGTAG	35
NT4	NT4AB-XHO-F	GACCTCGAG ACACGAACTTTGTCGATGCTGC	31
	NT4-BST-R	GCCCTTCGAATTTGCTACTCGTAGTCCTCCTC	32
	NT4C-XHO-F	GTCCTCGAGATGAGCAATGCGGGAATGGAC	30
	NT4BC-BST-R	TAAATTCGAAGACTTTTGGTAGTTCTTTACTCG	33

Mixtures of 20 µl were prepared for PCR, containing 13,6 µl Mili-Q water (Millipore), 2 µl exTaq Buffer, 1,6 µl dNTPs, 0,2 µl exTaq polymerase (Takara), 0,8 µl forward and reverse primer (Generi Biotech) and 1 µl DNA. Genomic DNA was used from yellow-white type (*yw*) of *Drosophila* and results confirmed also on Oregon wild type (OR). Additional mixture with previously optimized primers D1sarE and D1sarW from ADGF-A gene (634 bp product - genomic) was prepared as the control of the reaction. PCR program with 31 cycles was created (see Table 2) and ran on T3000 Thermocycler (Biometra).

Table 3: PCR program for primers optimization. * indicate variable annealing temperature, depending on the primer used.

step	temperature	time
1- initial denaturation	94°C	5 min
2 – denaturation	94°C	0 min 30s
3 – annealing	*	0 min 30s
4 – extension	72°C	4 min
5 - final extension	72°C	8 min
6 – hold	16°C	until pause
step 2 to 4 – repeated 30 times		

Gel electrophoresis was performed with obtained PCR products. 30 ml of 1% agarose gel were prepared from Agarose Serva Premium and 1x TAE buffer (Tris-acetate-EDTA buffer). Solidified gel was placed into electrophoretic vessel, which was filed with buffer afterwards. PCR products were centrifuged shortly and mixed with 10x loading dye. Approximately half of each reaction (9-10 µl) was loaded to the wells in the gel. 4 µl 1 kb ladder (New England Biolabs) was used as a marker. Electrodes were connected to the source and electrophoresis was run at 60 to 80 V. Finally, the gel was stained with ethidium bromide, washed, illuminated by UV light using transilluminator and a snapshot was taken.

3.2 DEVELOPMENTAL PROFILE CONSTRUCTION

Total RNA from 10 developmental stages of *Drosophila melanogaster* was isolated. mRNA part was transcribed to cDNA by a reverse transcriptase (SuperScript III Reverse Transcriptase, Invitrogen) and oligo dT primer. Newly synthesized first cDNA strands were amplified by PCR. The proper amount of cDNA for the PCR was established by running the reaction with ribosomal protein coding gene (rp49) primers. Such volumes of cDNA must have been used that the expression was the same in all developmental stages. The established amounts of cDNAs were used afterwards for PCR of the genes under investigation.

30 µl reactions were prepared. A master mix was made from 3 µl dNTPs, 1 µl of forward and reverse primer, 3µl exTaq Buffer and 0,25 µl of exTaq polymerase (Takara). cDNA was added according to established amount for the particular developmental stage and PCR water filled up to 30 µl. Additionally, a mixture with genomic DNA (Oregon) was prepared as

positive control. All genes were amplified in T3000 Thermocycler (Biometra) at 24, 30 and 36 cycles (see Table 3).

Table 4: PCR program for cDNA. * are variable values- annealing temperature depending on primers and extension time depending on sequence length (extension speed: 1kbp/min).

step	temperature	time
1- initial denaturation	94°C	5 min
2 - denaturation	94°C	0 min 30s
3 – annealing	*	0 min 30s
4 – extension	72°C	*
5 – hold	16°C	until pause
step 2 to 4 – repeated 23, 29 and 35 times		

Gel electrophoresis followed using 100 ml 1% agarose (Agarose Serva Premium) gel. PCR products were mixed with 5,8 µl 6x loading dye and 13 µl loaded on the gel. 4 µl 1 kb ladder (New England Biolabs) was used as a marker. Applied voltage started on 40 V and was switched to 65 V after a while. Pictures of the gels were taken.

3.3 PCR OF cDNA SAMPLES BY PROOF-READING POLYMERASE

One stage was chosen from each alternative transcript of the genes, where the gene was sufficiently expressed. New PCR was performed with cDNA of that stage using proof-reading polymerase. 50 µl mixture was prepared, containing 1 µl 10 mM dNTPs, 2,5 µl forward and reverse primer (Generi Biotech), 10 µl Phusion Buffer, 0,5 µl Phusion Polymerase (Finnzymes), cDNA according to established amount and PCR water up to 50 µl. Program was created (Table 4) on T Gradient machine (Biometra). 5 µl of PCR product were used for electrophoresis and the rest (45 µl) was purified and stored.

Table 5: PCR of cDNA with proof-reading polymerase (extension speed: 1kbp/30s).

step	temperature	time
1- initial denaturation	98°C	0 min 30 s
2 - denaturation	98°C	0 min 10s
3 – annealing	62,5 °C	0 min 30s
4 – extension	72°C	1 min 30 s
5 - final extension	72°C	8 min
6 – hold	14°C	until pause
step 2 to 4 – repeated 35 times, gradient: 9°C		

3.4 PCR PRODUCT PURIFICATION

Invisorb Fragment Clean Up 50 (Invitek) was used for purification of PCR product. 10 µl PCR product were mixed with 250 µl binding buffer, transferred to spin filter and centrifuged at 12000 rpm for 3 min. DNA was then eluted from the filter by 20 µl elution buffer, incubated for 1 min at RT and centrifuged at 12000 rpm for 1 min.

3.5 PREPARATION OF PCR PRODUCT FOR SEQUENCING

One gene (NT2) which yielded misleading bands was sent for sequencing. 20 ng of purified PCR product were mixed with 2,5 pmol primer and water was added to final volume of 7,5 µl. Two reactions were prepared, one with forward and the other with reverse primer. The results were compared with database of genomes by using BLAST search.

4 RESULTS and DISCUSSION

4.1 OPTIMIZATION OF PRIMERS

List of temperatures used for amplification of 7 alternative trascripts of the genes is given in Table 6. The temperature which showed the brightest and sharpest band on the gel was picked up or a temperature near to that one.

Table 6: List of temperatures for primers optimization

transcript	NT1	NT1 new	NT2	NT3B	NT3B new	NT3C	NT4A	NT4B	NT4C
t_1 (°C)	59	53	58	58	54	59	58	58	59
t_2 (°C)	62	55	60	60	57	62	60	60	62
t_3 (°C)	65	57	62	62	64	65	62	62	65
t_{final}	**-**	**55**	**62**	**-**	**64**	**64**	**62**	**62**	**64**

In NT1, temperatures which were set first did not give any bands, thus a new triplet of temperatures was empirically determined and PCR was repeated. It turned out that NT1 need lower annealing temperature. The same procedure was done with NT3. In this case, the second try worked for wide temperature range, indicating a preparation mistake in first PCR. Missing bands in NT1 and NT3 are caused by damage of the wells of the gel. PCR at 58°C yielded no band, which can be explained either by the fact that the temperature is too low for the particular genes or it was caused by some PCR mistake. The amplification worked well with other transcripts. Images of the gels are summarized in Figure 3.

Concerning the length of obtained sequences, all were correct when using yw genomic DNA for primers optimization (Figure 3). The result confirmation on Oregon DNA (Figure 4) brought one false result – NT2 showed a non-specific band. Instead of approx. 2100 bp, the sequence had more than 4500 bp. For this gene, mixtures were prepared again with freshly prepared primers and amplification was repeated. Both correct and incorrect band were visible on with yw DNA, the latter was less intensive. With Oregon DNA, just the false band was obtained (Figure 5).

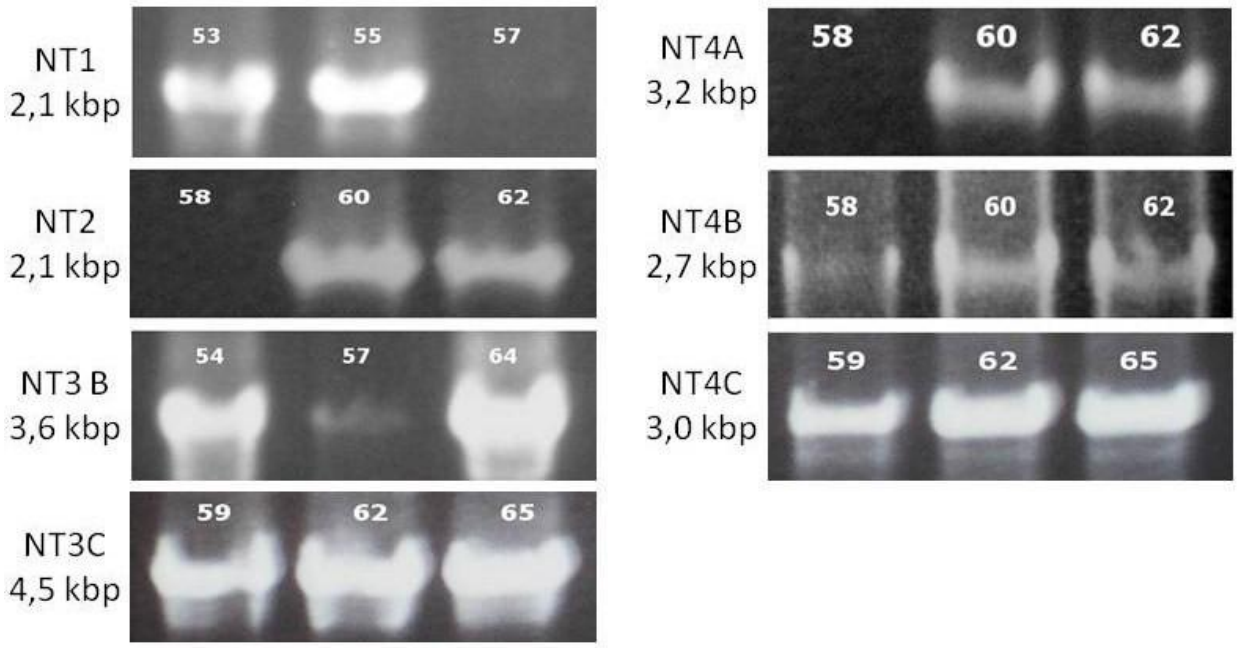

Figure 3: Optimization of primers - yellow-white DNA. Approximate length of sequences and annealing temperatures are included.

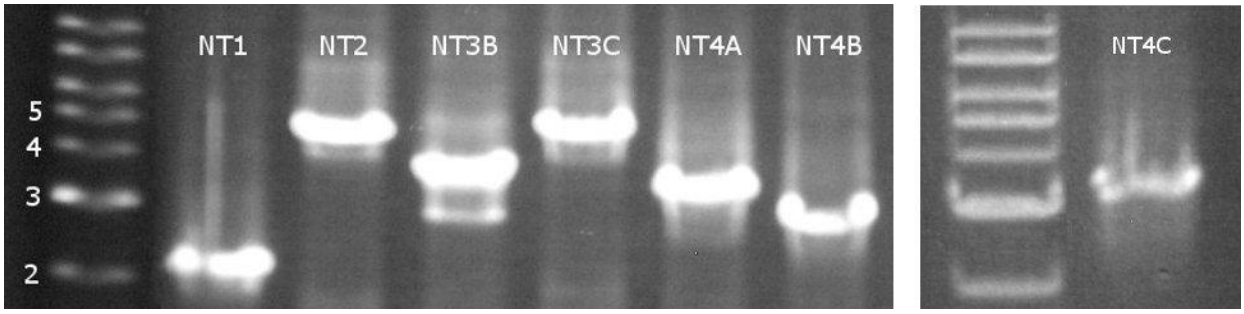

Figure 4: Optimization of primers - Oregon DNA. The NT2 sequence of a wrong length (4,5 kbp), NT4C reaction added separately due to repetition of experiment .

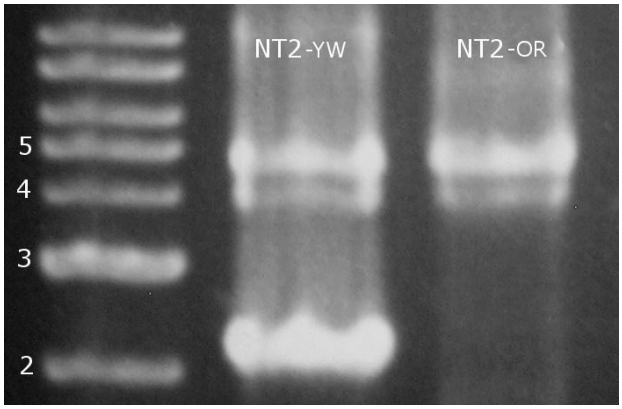

Figure 5: NT2 amplification – OR and YW DNA

Sequencing of the PCR product of NT2 (Oregon DNA) and comparison with genomes by BLAST (FlyBase) showed that forward primer recognized the correct gene, but reverse primer did not. Sequence from a completely different region in genome (chromozome x) was detected, where no gene is predicted (Figure 6). The explanation can be that we were dealing with a non-functional pseudogene which was derived from NT1 by duplication and sequence modification. This statement can be supported by the same length of the two sequences and proximity of both genes on chromosome 2. Further examination of the gene is required, e.g. running the reactions with newly designed primers.

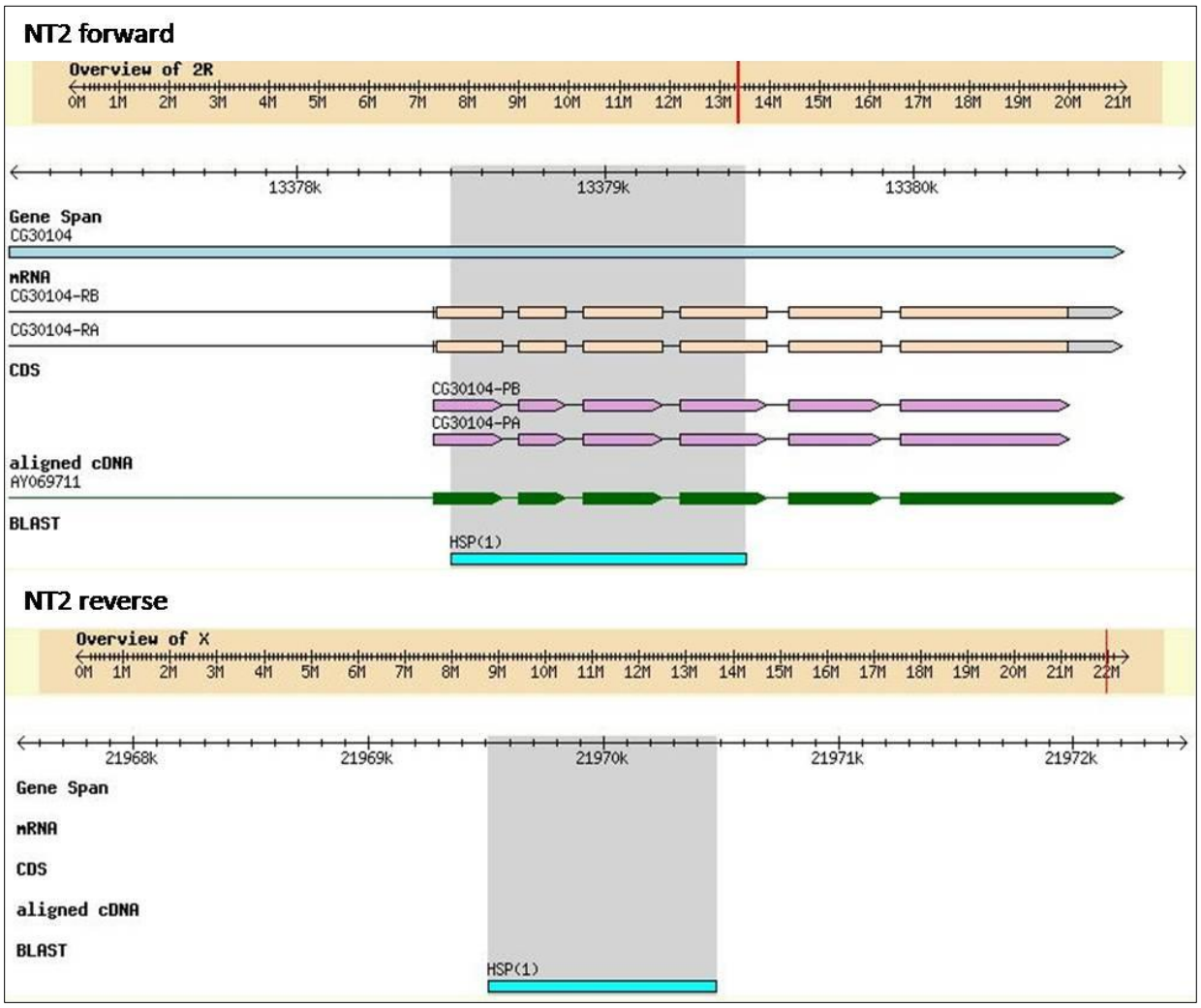

Figure 6: BLAST results of sequenced PCR product containing NT2F/NT2R primer. CG 30104 (NT2) was identified by forward primer, no relevant result was found for reaction with reverse primer.

4.2 DEVELOPMENTAL PROFILE

4.2.1 Rp49 amplification

The amount of cDNA for PCR reaction was adjusted for every developmental stage using control gene rp49, which is expected to be present in all cells in approximately the same amount. It was amplified by PCR with 20 and 22 cycles (Figure 7).

There was lower expression of cDNA of late embryos (Figure 7, #9) when using the amount around 1 µl. Higher volumes of cDNA (3 and 4 µl) had to be set empirically to get the band with the same intensity as other cDNA samples. Finally, 3 µl were found sufficient and used for experiments with NT genes. Because the RNA was isolated from Oregon type flies, the problematic NT2 gene was excluded from the investigation.

Table 7: Developmental stages of Drosophila and corresponding cDNA amount used for PCR

developmental stage	RNA concentration (µg/µl)	volume cDNA (µl)
embryo (0-6 h)	0,98	1
embryo (12-24 h)	0,86	3
½ 1st instar larva (36 h)	0,59	1
½ 2nd instar larva (60 h)	0,84	1
early 3rd instar larva (84 h)	1,33	1
3rd instar larva - wandering	1,15	1
prepupa	0,71	1,2
pupa (26 h)	1,17	2
adult male (3 days)	0,32	1,7
adult female (3 days)	0,53	1

Figure 7 : RP49 amplification – results after optimization for 20 and 22 cycles. Labelling: 1= adult male, 2= adult female, 3 = pupa, 4= prepupa, 5= 3rd instar larva wandering, 6= 3rd instar larva, 7= 2nd instar larva, 8= 1st instar larva, 9= late embryo, 10= early embryo

4.2.2 Developmental profile of ectonucleotidase genes

Amplification of the genes was done at 24, 30 and 36 cycles. Out of that, 30 or 36 cycles were picked up for evaluation of gene activity. Nothing was visible after 24 cycles. cDNA of late embryos (12-24h) was not amplified in any of the developmental stages. That is quite improbable when we take into account that all the other cDNAs worked well, i.e. were amplified at least in one stage. It was concluded that the negative result is due to bad cDNA, resulting from a mistake in RNA isolation or reverse transcription. The cDNA was removed from the gel images. The profiles of NT genes are depicted in Figure 8.

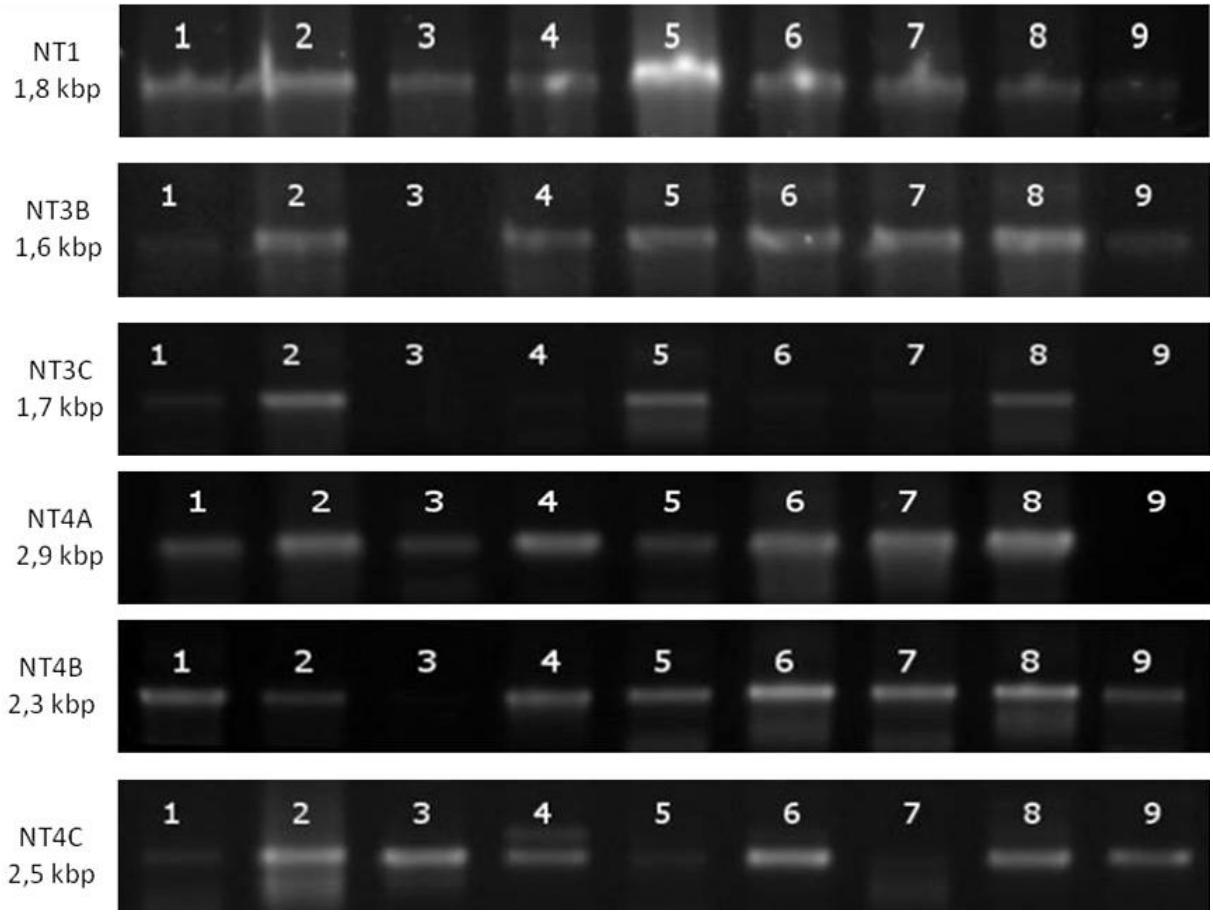

Figure 8: Developmental profile of NT genes, including approximate lengths of sequences. Labelling: 1= adult male, 2= adult female, 3 = pupa, 4= prepupa, 5= 3rd instar wandering, 6= 3rd instar, 7= 2nd instar, 8= 1st instar larva, 9= early embryo

NT1 gene was expressed in all stages, less in female adult (1), pupa(3) and embryo (9), on the other hand little more in male adult (3) and larvae (5-8).

NT3B transcript was expressed in all stages except one. In pupa (3), there is no visible band, and in female adult (1) and embryo (9), it is little gene activity.

NT3C was transcribed in adults (1,2), 3rd instar wandering (5) and 1st instar larvae (8), nearly invisible bands can be seen also in prepupa (4) and rest of larval stages (6,7). No bands are in pupa (3) and embryo stage (9).

NT4A was expressed in all developmental stages except of early embryos (9). More visible are the bands of larval stages (6-8) and male adult (2).

NT4B was expressed in all stages. However, the band of pupa(3) is very poorly visible. Very bright are the bands of larval stages (6-8).

NT4C was expressed in all stages, little in adult female (1), 3rd instar wandering and 2nd instar (5,7) and pretty much in adult male (2), pupa (3) and 3rd instar larva (6). Some unspecific bands are visible in 4 to 7, that are probably due to other proteins present in the reaction.

The obtained profiles are in accordance with developmental profiles from FlyBase. RNA-seq provide information about expression of regions of mRNA, while in our work the expression is investigated over the whole transcript. However, it is possible to compare the intensity of expression in the two profiles. As an example, the RNA developmental profile of NT1 was taken (Figure 9). We can see lower expression in embryonal and pupal stages (E,P) and higher in adults and larvae (L,A), which corresponds to the results obtained from cDNA amplification of NT1.

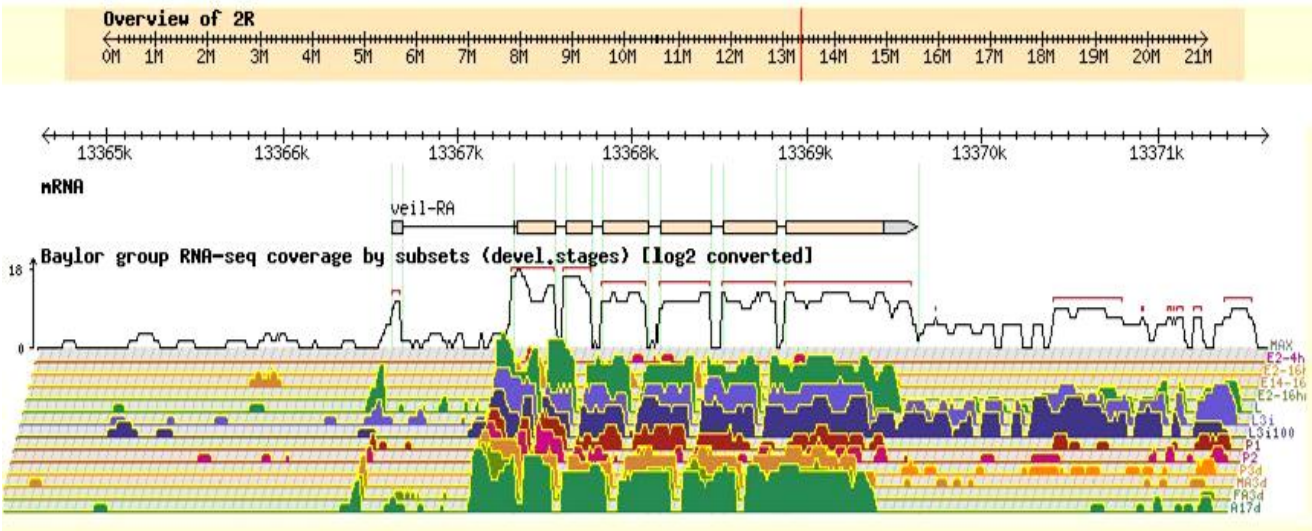

Figure 9: **RNA-seq developmental profile of CG4827 (NT1)**

4.3 PCR OF SELECTED cDNA SAMPLES BY PROOF-READING POLYMERASE

cDNA of the stage with relatively stronger expression was chosen for each NT gene. PCR employing proof-reading polymerase (Pfu) instead of ordinary one was performed and PCR products were then purified. After such pre-treatment, they were ready to be cloned. The list of selected cDNAs with relevant information for further work is given in Table 8. Gels of amplified cDNAs before and after cleaning are displayed in Figure 10.

Due to a mistake in calculation, NT4A gene first seemed to be of a wrong length, therefore it was omitted from proof-reading amplification. After recalculations it showed up that the length was correct. The procedure for NT4A will be completed before the start of cloning.

Table 8: Selected stages and restriciton enzymes used for cloning

transcript	selected stage	5´ restriction enzyme	3´ restriction enzyme
NT1	2nd instar larva (60 h)	*Not* I	*Bst*B I
NT3B	1st instar larva (36 h)	*Eco*R I	*Bst*B I
NT3C	adult male (3 days)	*Eco*R I	*Bst*B I
NT4B	adult female (3 days)	*Xho* I	*Bst*B I
NT4C	3rd instar larva (84 h)	*Xho* I	*Bst*B I

- Not = *Nocardia otitidis-caviarum*
- Eco = *Escherechia coli*
- Xho = *Xanthomonas holcicola*
- Bst = *Bacillus stearothermophilus*

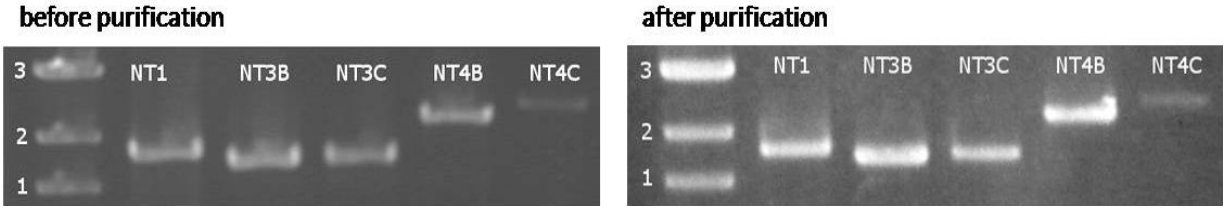

Figure 10: Amplification of cDNA by Pfu polymerase - before/after PCR product purification

5 CONCLUSION

As the first step of the work, optimal annealing temperatures of designed primers was determined and the length of NT sequences was checked. There appeared some inconsistencies in optimization of primers for NT2. For that reason it was excluded from further cDNA experiments.

RNA from 10 developmental stages of *Drosophila* was isolated, transcribed to cDNA and amplified. The expression of predicted ecto-5´-nucleotidase genes NT1, NT3 and NT4 was recorded in all developmental stages of *Drosophila melanogaster*. The intensity of gene expression at the transcription level differed in particular stages. Some general observations could have been deduced from the images of the gels. First of all, the genes were more expressed in the male than the female adults. In pupae, gene expression was very low, NT3 showed no expression at all. The gene activity in most prepupal stage and all larval stages was found pretty high. Lower expression can be seen only in some larvae (NT3C and NT4C). In embryos, the observed NT activity is low except NT4 B and C gene. The obtained profiles are in good agreement with developmental profiles from FlyBase.

5 different transcripts of 3 out of 4 genes with putative ectonucleotidase activity are now prepared for cloning and subsequent functional studies. The purified PCR products of chosen stages will be inserted into a cloning vector, multiplied in bacteria and transfected into S2 cells of *Drosophila melanogaster* (embryonic blood cells). Actin promoter will be also present in the cloning vector to initiate a strong expression in S2 cells. The ectonucleotidase activity after the induction of expression will be then investigated in the cell culture. Isotopically labelled AMP will be added to the medium and the amount of labelled adenosine will be subsequently measured by time-of-flight mass spectrometry. If there is a significant increase of adenosine made from labeled AMP compared to a basal level, the ecto-5‘-nucleotidase activity of the protein will be confirmed. Another procedure involves a measurement of a pyrophosphate generated during the reaction by simple measurement kit.

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