Structural and functional investigation of the *Drosophila melanogaster Ada2a/Rpb4* and *Dtl* genes

PhD Thesis

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Introduction

My PhD. thesis is on the structural of functional analysis of two Drosophila genes, which encode proteins involved in transcription regulation. We became interested in these genes several years ago when we identified them and learned that their protein products are RNA polymerase II subunit, transcription coactivator, and a novel protein which shows structural similarities to the transcriptional activator of Human Immunodeficiency Virus.

Regulation of gene expression in eukaryotic cells is a complex process which is tightly regulated at several steps. Transcription, and particularly transcription initiation, the first step of gene expression itself is a target of diverse regulatory mechanisms. For the start of RNA synthesis the RNA polymerase II (pol II) and several so called basal transcription factors need to assemble at the 5' region of a gene forming the preinitiation complex. The basal factors help the polymerase to locate the promoter and initiation site. The binding sequences of these factors are located usually within a 100 bp short region and constitute the so-called basal promoter. The activity of pol II and basal transcription factors is influenced by transcriptional activators and repressors through they binding to specific sequences located further away from the site of RNA initiation. Some of the regulatory elements might be located several thousand base pairs away from the basal promoter in either direction. Additional complexity of the transcription regulation arises from the chromatin structure: the activity of specific mediator and co-activator complexes are needed to ensure that a given gene is accessible to the basal transcription machinery and sequence specific activators. Finally, gene expression regulation can take place even after transcription is completed: the stability of mRNA, activity of factors which degrade specific mRNAs selectively, efficiency of translation and several other mechanisms influence the expression of a given gene.

Aims

The two genes I describe in my thesis were identified at the time I started my Ph.D work. *Dtl* (Drosophila Tat-like) was identified based on it similarity to the HIV Tat protein. We started studying it to determine whether a regulatory mechanism known to exist only in a virus can be found *Drosophila* too. Later we learned that *Dtl* is closely associated with *Ada2/Rpb4*, which itself deserves attention as a transcription unit that gives rise to two important transcriptional factors, an RNA pol II subunit (RPB4) and a co-activator (ADA2a). None of these genes were known in *Drosophila* at that time, neither was the Drosophila genome project completed yet.

Therefore, my first aim was to describe the structural organization of the *Dtl* and *Ada2a/Rpb4* genes and their relation to each other.

As work on the *Dtl* and *Ada2a/Rpb4* genes progressed we realised they close association and a detailed analysis of their regulation, identification and characterization of their promoters became necessary.

At the latest part of my work I participated in studies on the functional analysis of *Dtl*, with the aim to determine the biological activity its protein product is responsible for.

Methods

- DNA modification techniques
- Total RNA preparation from adult *Drosophila*
- 5' RACE
- Southern hybridisation
- RT-PCR
- Staining *Drosophila* tissues
- Transfecting Schneider S2 cells and luminescence measuring
- Nuclear extract preparation from Schneider S2 cells
- Electromobility Shift Assay (EMSA)
- Total protein extract preparation from *Drosophila* embryos
- RNA stability Assay
- Formaldehyde gelelectrophoresis
- RNase Protection Assay
- Denaturing polyacrylamide gelelectrophoresis
- Immunoprecipitation
- Northern hybridisation

Results and discussion

Structural analysis of the Drosophila melanogaster Dtl and Ada2a/Rpb4 genes

I investigated in my thesis two *Drosophila* genes located on the 3rd chromosome in the 90F9-11 cytologic region. At the beginning of the work neither of them were identified, so we had the opportunity to describe them first. Using primer extensions and 5'RACE we identified the exact location and exon-intron structure of the genes. We named them *Dtl* and *Ada2a/Rpb4* based on

homologues. We found that two mRNAs are transcribed from the *Ada2a/Rpb4* via alternative splicing coding the ADA2a and the RPB4 proteins. We also discovered that the *Dtl* gene consists of two Open Reading Frames.

We identified the transcription start site of the two genes, and they are located only 73 bps from each other. According to the literature an average Drosophila promoter extends from 80 to several hundreds of bps. The close proximity of the genes raises the possibility of the overlap of the two promoters. In this case it is possible that the regulatory elements of one gene are located in the coding region of the other gene. Therefore we mapped the cis-regulation element of the genes using transgenic flies and luciferase assays in tissue cultures. The analysis of the promoter of the *Ada2a/Rpb4* gene showed that a short, 79 bp long promoter fragment carries all the elements needed for the proper transcription initiation. This fragment shows a specific expression pattern in transgenic animals: in larvae we detected expression using LacZ-fusion construct in the central nervous system, in the testis and in the imaginal discs, while only the gonads of the adult Drosophila showed LacZ expression. Since ADA2a is a general coactivator and RPB4 is a subunit of the RNA polymerase II, we suppose that the observed expression pattern is due to the lack of other tissue-specific regulatory regions. On the other hand the expression of LacZ in the transgenic flies proved that this short promoter fragment contains the necessary elements for transcription initiation.

By analysing the *Dtl* promoter in S2 cells we concluded that there are elements influencing transcription on the analysed promoter fragments. Using luciferase reporter assays we mapped these elements to the (-412)-(-616) region from the transcription start site of the *Dtl* gene. These regulatory sequences are located in the coding region of the *Ada2a/Rpb4* gene. Promoter elements located in the coding region usually take part in RNA maturation instead of transcription initiation. We also investigated the *Dtl* promoter using transgenic flies. We used two promoter fragments, one that had the whole regulatory region (the one we

analysed in S2 cells), and one that was only 180 bps long. We detected tissue-specific expression using these two fragments like in the case of *Ada2a/Rpb4*. We found differences between the expressions of the two promoter fragments. The longer fragment showed weaker expression in the larval gut and the salivary glands compared to the shorter fragment. The observed difference may be due to a tissue specific repressor seating on the longer promoter fragment.

According to the experiments in S2 cells we predicted that the *Dtl* promoter region overlaps with the *Ada2a/Rpb4* coding region. To support this theory we performed electromobility shift assays (EMSAs). We could detect specific DNA-protein complexes in the *Ada2a/Rpb4* coding region and in the intergenic region between *Dtl* and *Ada2a/Rpb4*. *In silico* analysis revealed three potential DNA binding factor sites in the *Ada2a/Rpb4* coding region: Fushi Tarazu, Tramtrack and Suppressor of Hairless. The role of these proteins in gene expression regulation is not clarified yet.

We detected specific protein-DNA complexes between the transcription start sites of the two genes. Using synthetic oligonucleotides and α-DREF antibody we demonstrated that the altered DNA mobility is due to a sequence element (DRE) usually seating in the promoters of genes playing a role in the chromosome replication and proliferation. With the help of mutant DRE containing promoter constructs we concluded that this DRE plays a role in the *Ada2a/Rpb4* expression and has no effect in the *Dtl* regulation in S2 cells.

The posttranscriptional regulation of the Dtl and Ada2a/Rpb4

Using RNase protection assays we detected an RNA in the last four exon of the *Ada2a/Rpb4* gene transcribing in the opposite direction. *In vitro* transcribed radioactive RNA coming from this region showed specific degradation in Drosophila embryo extract. Further investigations revealed that the observed degradation is executed by a high molecular-weight enzyme complex. On the

basis of these results we predicted that RNA interference plays a role in the observed RNA degradation.

Functional analysis of the Dtl gene

To understand the function of the DTL protein we made deletion mutant flies. These animals die at L2-L3 larval stage in the absence of the DTL protein. Using immunohistochemistry and immunoprecipitations we observed aberrant chromosome segregation during mitosis and altered cap structure of the sn and snoRNAs: the trimethylation is absent. With the help of rescue experiments we concluded that the observed phenotype can be linked not only to the absence of the DTL protein, but also the lack of an ORF located in the 5' UTR (DTLu). The DTL protein consists of two domains, an RNA methylation and an RNA binding domain. The role of these two domains in the observed mutant phenotype is not clarified yet. The DTLu has only one homologue in the *Drosophila pseudoobscura*, its function is unknown.

Short summary of results:

1, By the use of different techniques of transcript mapping I characterised the divergently transcribed *Dtl* and *Ada2a/Rpb4* genes of Drosophila and determined that they have overlapping transcription regulatory region which might suggest their functional linkage.
2, Using *in vitro* and *in vivo* promoter mapping techniques and transgenic flies which carry promoter-reporter gene constructs, I completed a detailed promoter analysis of the *Dtl* and *Ada2/Rpb4* genes, identified the cis-regulatory elements in those and some of the trans-acting factors involved the transcription of these genes.
3, I demonstrated that at some regions of the *Ada2a/Rpb4* gene transcription proceeds in both directions, and showed that this could result in specific degradation of the Ada2a and the Rpb4 mRNAs, which could be a further regulatory mechanism acting on these genes.
4, By constructing specific *Dtl* transgenes and analysing animals carrying those I determined that the product of *Dtl* is essential for the formation of specific cap structure of small nuclear and nucleolar RNAs in *Drosophila*.

Together these results contributed significantly to the molecular characterization of two newly identified *Drosophila* genes with important functions in transcription, provided new data on the organization of transcription regulatory elements, and on the functional role of trimethylguanosine synthase required for sn- and snoRNA cap formation.

Publications, presentations

List of publications

Rakonczay Z. Jr, Takács T., Mándi Y., Iványi B., Varga I., **Pápai G.**, Boros I., Lonovics J. Water immersion pretreatment decreases pro-inflammatory cytokine production in cholecystokinin-octapeptide-induced acute pancreatitis in rats: possible role of HSP72. Int J Hyperthermia 2001, 17: 520-535

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