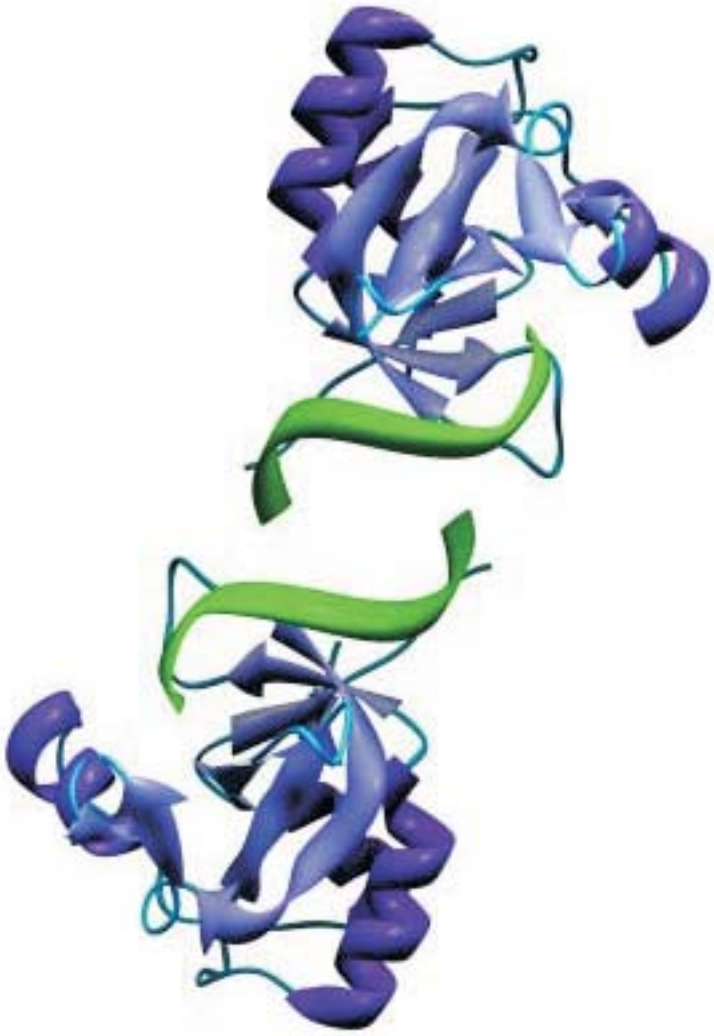
RNA Club



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České Budějovice

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Katedra molekulární biologie
Branišovská 31
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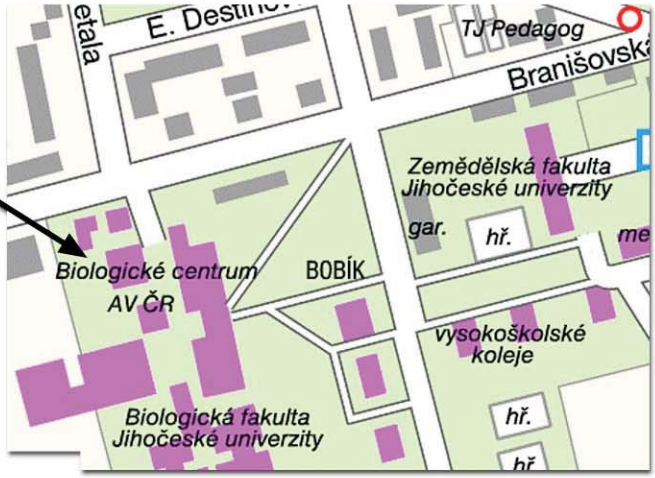
3rd RNA CLUB
3 October 2005

Conference Venue:
Building "B"
Biological Faculty
University of South Bohemia
Branišovská 31
České Budějovice

Opening: 9 am



Campus map

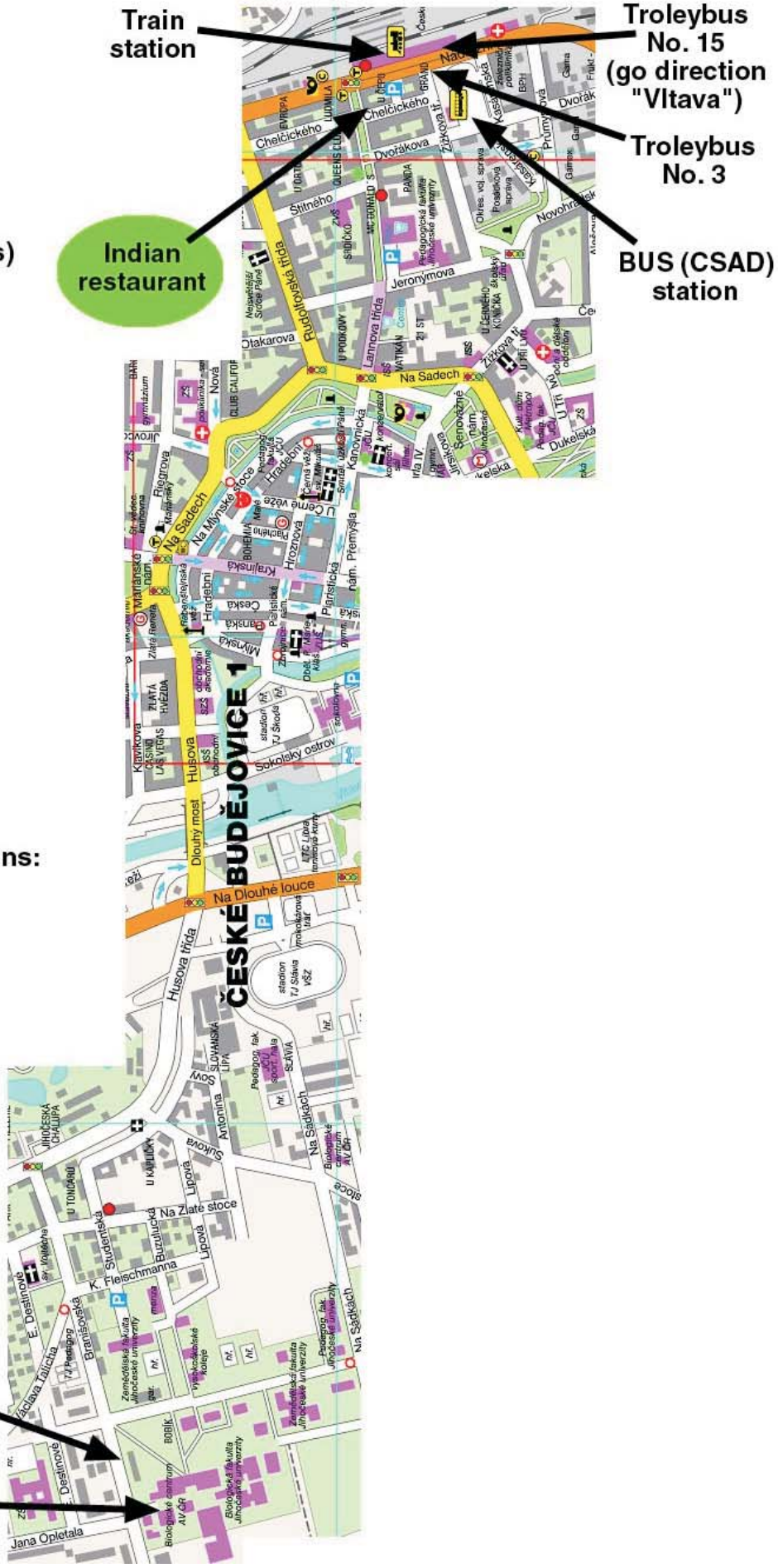
Main entrance

Building "B" (conference site)

To get to RNA Club at the Biological Faculty:

take troleybus No. 3 or 15 from locations near Train/Bus stations (indicated by arrows)

get off at "Jana Opletala"



To get to Indian restaurant and Train/Bus stations:

take troleybus No. 3 or 15 from "Jana Opletala" (runs every 10 min)

PROGRAM

9:00 **REGISTRATION**, installation of posters
9:40 Announcement by the organizers
9:50 Opening address of the Dean, Prof. Libor Grubhoffer

SESSION 1: Structure and Function of RNA Molecules
(Chair: Martin Pospíšek)

10:00 **Leoš Valášek:** Interactions of eIF3 Subunit NIP1 with eIF1 and eIF5 Promote Preinitiation Complex Assembly and Regulate Start Codon Selection
10:30 **Libor Krásný**: Regulation of ribosome synthesis in bacteria
10:45 **Zuzana Feketová**: ORF 19.7626 from *C. albicans* functionally complements the *cdc33* mutation in *S. cerevisiae*
11:00 **Silvie Trantírková** : Functional analysis of proteins involved in trans-splicing in *Trypanosoma brucei*
11:15 **Zdeněk Chval**: A mechanism of the O2' activation step for hammerhead ribozyme catalysis – a theoretical study

11:30-12:00 **Presentations of sponsoring vendors**
11:30-11:40 **Roche:** The Universal ProbeLibrary for qPCR
Other vendors: 5 minutes per presentation

12:00-13:30 Lunch Break, Poster Discussions, and Vendor Exhibitions

SESSION 2: Cell and Developmental Biology
(Chair: Marek Jindra)

13:30 **Gregor Bucher:** How does a beetle get its head: RNAi tells us
14:00 **David Staněk**: Spliceosomal snRNP assembly in the cell nucleus
14:15 **Vendula Macečková**: Addressing the role of vimentin in monocyte/macrophage differentiation using RNA interference approach
14:30 **Júlia Starková**: The expression of genes associated with TEL/AML1[+] leukaemias is changed by histone deacetylase inhibitors
14:45 **Daniel Růžek**: Identification of two potential molecular determinants of an attenuated temperature-sensitive phenotype of tick-borne encephalitis virus
15:00 **David Honys**: Male gametophyte development: a transcriptomic view

15:15-15:45 Coffee Break, Poster Discussions, and Vendor Exhibitions

SESSION 3: RNAi and Other Techniques
(Chair: Lukáš Trantírek)

15:45 **Julius Lukeš:** RNAi-based functional study of RNA editing in kinetoplastids
16:15 **Markéta Žaliová**: Silencing of ETV6/RUNX1 fusion mRNA using siRNA
16:30 **Aleš Neuwirth**: In vitro suppression of inducible NO synthase expression using siRNA
16:45 **Ema Ruzsová**: Arrays – non-invasively directly from the skin
17:00 **Nikoleta Dupľáková**: Arabidopsis Gene Family Profiler – a new easy-to-use family-oriented gene expression database
17:15 **Helena Štorchová**: The analysis of plant mitochondrial transcripts by means of non-radioactive Northern hybridisation and quantitative RT PCR

18:00- Dinner at the Indian restaurant (please see **Map** for directions)

ORAL PRESENTATIONS

10:00

Interactions of eIF3 Subunit NIP1 with eIF1 and eIF5 Promote Preinitiation Complex Assembly and Regulate Start Codon Selection

LEOŠ VALÁŠEK[1,2], NIELSEN KH[2], ZHANG F[2], FEKETE CA[2], HINNEBUSCH AG[2]

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Translation initiation is a multiple-step process involving a large number of soluble eukaryotic initiation factors (eIFs). Among them, eIF3 draws a special attention as it was shown to promote several critical steps of the initiation pathway mainly the recruitment of the eIF2.GTP.Met-tRNA$_i^{Met}$ ternary complex (TC) and mRNA to the 40S ribosomal subunit. We demonstrated that the N-terminal domain (NTD) of the NIP1 subunit of eIF3 interacts directly with eIF1 and eIF5, and indirectly through eIF5 with the eIF2/TC, to form the multifactor complex (MFC) that is an important intermediate of the pathway. Interestingly, the latter three factors were implicated in the stringent selection of the AUG start codon. We investigated the physiological importance of these NIP1-mediated interactions by mutating 16 segments spanning the NIP1-NTD. Mutations in multiple segments reduced the binding of eIF1 or eIF5 to the NIP1-NTD. Mutating a C-terminal segment of the NIP1-NTD increased utilization of UUG start codons (Sui$^-$ phenotype) and was lethal in cells expressing eIF5-G31R that is hyperactive in stimulating GTP hydrolysis by the TC at AUG codons. Both effects of this NIP1 mutation were suppressed by eIF1 overexpression, as was the Sui$^-$ phenotype conferred by eIF5-G31R. Mutations in two N-terminal segments suppressed the Sui$^-$ phenotypes produced by the eIF1-D83G and eIF5-G31R mutations. From these and other findings, we propose that the NIP1-NTD dovetails an interaction between eIF1 and eIF5 that inhibits GTP hydrolysis at non-AUG codons. To conclude, we propose that the NIP1-NTD is required for efficient assembly of pre-initiation complexes and seems to coordinate the functions of eIF1, eIF5, and TC in the AUG recognition process during scanning. The current view of the translation initiation pathway will be presented.

10:30

Regulation of ribosome synthesis in bacteria

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Rapidly growing bacteria need a high number of ribosomes to satisfy the cell's need for increased translation. Starving bacteria need a low number of ribosomes to limit energy expenditure. The number of ribosomes is regulated at the level of ribosomal RNA (rRNA) transcription.

Escherichia coli rRNA transcription regulation has been studied for decades. I will present studies on gram positive *Bacillus subtilis* rRNA transcription regulation. The data suggest that B. subtilis has solved the regulation in a mechanistically different way than E. coli (Krasny and Gourse, EMBO J 2004, 23:4473-83).

10:45

ORF 19.7626 from *C. albicans* functionally complements the *cdc33* mutation in *S. cerevisiae*

ZUZANA FEKETOVÁ[1], MAŠEK T[1], VOPÁLENSKÝ V[1], POSPÍŠEK M[1,2]

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The eIF4E factor is one of the main regulators of translation initiation affecting overall translational rates. *C. albicans* seems to be unique in the otherwise evolutionary well conserved process of eukaryotic translation initiation since this organism is able to survive as a null mutant in two of three 7mG cap-synthesising enzymes (Dunyak et al., 2002). These mutants are apparently able to form and recognize modified cap structures. To further develop and test the idea of possible unique features of the *C. albicans* translation initiation machinery we prepared *S. cerevisiae* strains dependent solely on the putative *C. albicans* eIF4E translation factor. Growth analyses, polysomal profiles and immunochemical analyses of these strains were performed and revealed high level of heterologous production of *CaCDC33*, temperature sensitivity of these strains and affected polysomal profiles. This phenotype might originate from differences in codon usage between both yeast species, weaker interactions with binding partners or from an intrinsic feature of the *CaCDC33*.
This work was supported by GACR - Grant No. 204/03/1487, by GA UK - Grant No. 190/2005/B-BIO/PrF and by the Ministry of Education (Grant No. MSM 0021620813)

Literature: Dunyak DS, Everdeen DS, Albanese JG, Quinn ChL. (2002) Eukaryot Cell. 1(6): 1010-20

11:00

Functional analysis of proteins involved in trans-splicing in *Trypanosoma brucei*

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Trypanosomes are early-branching eukaryotes with a number of unique features, a prominent one being trans-splicing. Trans-splicing in T. brucei is an essential step in the maturation of all mRNAs. It results in joining of a short, non-coding spliced leader RNA (SL RNA) sequence to protein-coding sequences that are transcribed as polycistronic precursors. The essential cofactors for trans-splicing are, in addition to SL RNA, small nuclear RNAs termed U2, U4, U6 and U5. Each of these RNAs has to undergo characteristic maturation to create a trans-splicing competent form. Using RNA interference, we have studied the function of several proteins involved in trans-splicing, namely SmD1, La, methyltransferases and TSR1.We have shown that the SmD1 protein is essential for accurate SL RNA maturation, its absence resulting in aberrant 3' end processing, partial formation of cap4 and over-accumulation of SL RNA in the cytoplasm. Unexpectedly, although the La protein binds to SL RNA, it is not essential for its maturation. This, however, does not rule out possible existence of an alternative processing pathway that might compensate for the absence of the SL RNA-associated La protein. Currently, we are investigating the role of methyltransferases and the TSR1 protein for SL RNA maturation and trans-splicing of pre-mRNA, respectively.

(Foldynova-Trantirkova et al. (2005) Int J Parasitol 35:359-66; Ismaili et al. (1999) Mol Biochem Parasitol 102:103-15; Mandelboim et al. (2003) J Biol Chem 278:51469; Schneider et al. (1993) J Biol Chem 268:21868-74; Zeiner et al. (2003) Eukaryot Cell 3:241-4.

11:15

A mechanism of the O2' activation step for hammerhead ribozyme catalysis – a theoretical study

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The O2' activation (dehydrogenation of O2'-H group) of the C17 nucleotide is generally considered to be the first step in the phosphodiester hydrolytic cleavage reaction of the hammerhead ribozyme (HHR). Activated O2′ atom is then able to attack phosphorus atom and cleavage proceeds via five-coordinated transition state to form 2′,3′-cyclic phosphate diester. The mechanism of the cleavage and the number of metal ions involved are still not fully understood.
An idea proposed by Pontius et al.(1) is studied in this contribution. Due to high value of pKa of O2'-H hydroxide (14.9(2)), the O2' activation should be coupled with its direct binding to the metal ion(1). The mechanism proposed here includes the following reaction steps: 1) a lowering of the coordination number on the Mg2+ ion by expelling of one of water ligands to the second hydration shell. 2) Coordination of O2′-H group to the penta-coordinated Mg2+ ion, and 3) a hydrogen transfer from O2' to OH- group.
The reaction profile is calculated in the gas-phase and in solution using implicit solvation models. It is shown that under basic conditions the lowering of the coordination number on the magnesium atom from six to five is the rate-determining step for the O'2 activation with activation energy of 8.0 kcal/mol. The subsequent O2′-H coordination and the proton abstraction from O'2 need only small barriers of max. 2.5 kcal/mol. Our results fully confirm the hypothesis of Pontius, the metal ion coordination to 2'O substantially lowers the pKa value of the O'2-H group being estimated to be comparable to that of hexa-hydrated magnesium (11.4). Calculated activation energies are much lower than an experimental value of ~20 kcal/mol(3,4) determined for the barrier of the cleavage reaction. Mg2+ coordination to the O2′ oxygen can be expected as a highly probable event prior to HHR cleavage.
Results are fully consistent with the two-metal-ion model for the hammerhead ribozyme cleavage reaction.

1) Pontius, et al. (1997) Proc. Natl Acad. Sci. U.S.A. 94:2290
2) Lyne, et al. (2000) J. Am. Chem. Soc. 122:166
3) Uhlenbeck, (1987) Nature 328:596
4) Torres, et al. (2003) J. Am. Chem. Soc. 125:9861

11:30

The Universal ProbeLibrary for qPCR

HYNEK STODŮLKA

Roche s.r.o., Diagnostics division, Karlovo nám. 17, 120 00 Praha 2
(hynek.stodulka@roche.com)

Universal ProbeLibrary is new technology which redefining and revolutionizing real-time qPCR assays. There is no need waiting for prevalidated, specific probes. Specific real-time qPCR assays can be designed in 30 seconds and prices for this type of specific hydrolysis probes are comparable to SYBRGreen I. One standard PCR protocol can be used with dedicated master mix on any real-time PCR instrument.

Universal ProbeLibrary is 165 specific and prevalidated hydrolysis probes that can quantify virtually any transcript of a large number of organisms. Organism specific sets of 90 probes are available for: human, mouse, rat, primates, drosophila, C. elegans, or arabidopsis.
Primers and probes for the specific intron spanning assay are designed in the web-based Assay Design Center at *www.universalprobelibrary.com*

The unique versatility of the Universal ProbeLibrary probes has been achieved by shortening the length of the dual-labeled probes from 25-35 nucleotides to only 8-9. The sequences have been carefully selected to hybridize to the most prevalent sequences in the transcriptomes. Each probe can hybridize to over 7000 transcripts. Specificity is attained by the combination of primers and the probe. High melting temperature and specificity of the short real-time PCR detection probes is retained by using a proprietary nucleotide chemistry, called Locked Nucleic Acids (LNA).
The unique combination of software and 165 prevalidated probes enables the design of over two
million real-time PCR assays. Over one half million of these are intron-spanning assays.

More info *www.universalprobelibrary.com*

13:30

How does a beetle get its head: RNAi tells us

GREGOR BUCHER

Institute for Zoology, Anthropology and Dev. Biology
Dpt. for Developmental Biology
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Some genes involved in head development are conserved from mouse to fly. Nevertheless, head segmentation is not understood even in the fruit fly *Drosophila melanogaster* where markedly derived head morphology has hampered the analysis of mutants. We have established the red flour beetle *Tribolium castaneum* as a model system for head development. One reason is that its head develops in a way typical for insects. The other reason are the strong powerful reverse genetics that can be applied to *Tribolium*: Its genome is sequenced and gene function can be knocked down efficiently using RNA interference (RNAi). Embryonic injection of double stranded RNA can phenocopy the Null phenotypes of mutations. Furthermore, a technique called *parental RNAi* allows us to collect a large number of knock down offspring from injected female pupae. These embryos can be stained using standard procedures allowing for an in depth analysis of gene interactions.

I will introduce you to the design of our reverse genetics approach and to first results that we have obtained regarding head development. In addition, I will discuss advantages and disadvantages of RNAi in comparison to classical genetic approaches. Finally, the strong impact of RNAi and parental RNAi on evolutionary developmental studies will be emphasized.

14:00

Spliceosomal snRNP assembly in the cell nucleus

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[2]Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany

Cajal bodies (CBs) are 0.5-1.0 micron nuclear bodies found in various cell types and organisms. CBs are enriched in a subset of factors required for RNA processing and transcription, however the activities occurring in CBs are largely unknown. Here we investigated the interactions, the dynamics and the targeting of proteins involved in pre-mRNA processing. To examine a potential role of CBs in assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs), we investigated the distribution of specific snRNP intermediates containing the SART3 protein, which only transiently interacts with U6 and U4/U6 snRNPs and is required for U4/U6 snRNP assembly. Using fluorescence resonance energy transfer we detected SART3 containing snRNP intermediates. We found that SART3•U6 snRNP complexes are found exclusively in the nucleoplasm and SART3•U4/U6 snRNP preferentially in CBs. Using microinjection and expression of the dominant negative mutant of SART3 we also showed that U6 and U4 snRNPs are targeted independently to the CB supporting the model that U4/U6 snRNP assembly occurs in CBs.

14:15

Addressing the role of vimentin in monocyte/macrophage differentiation using RNA interference approach

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Changes of expression of many genes, including those coding for intermediate filament (IF) proteins, are essential for cell differentiation. The changes of gene expression are either qualitative causing a switch of expression from one type of IF to another or quantitative resulting in production of different amount of the same IF protein. Vimentin is one of the intermediate filament proteins that undergoes significant changes both in expression, structure and subcellular localization during maturation of hematopoietic cells of various lineages. Monocytes/macrophages are the only blood cells with fully developed vimentin network in cytoplasm.

The v-myb-transformed monoblasts of the cell line BM2 can be induced to differentiate towards macrophage-like cells using phorbol ester TPA and histon deacetylase inhibitor trichostatin A (TSA). We described earlier that vimentin level increases during TPA or TSA induced differentiation of BM2 cells using western blotting and indirect immunoflourescence. In this study, the vector-based siRNA technology was used to explore the role of vimentin in differentiation of BM2 cells. The cells were transfected with vector expressing siRNA targeted to endogenous vimentin mRNA and several independent clones of stable transfectants exhibiting 70% reduction of cellular vimentin level were obtained. The lack of vimentin altered cellular morphology, reduced their phagocytic activity and production of reactive oxygen species upon treatment with TPA or TSA.

Our results demonstrate that up-regulation of vimentin gene expression is essential for formation of fully active macrophages during terminal steps of monocyte/macrophage differentiation.

14:30

The expression of genes associated with TEL/AML1 [+] leukaemias is changed by histone deacetylase inhibitors

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The role of TEL/AML1 fusion gene in leukaemogenesis is still not understood despite the frequent occurrence in children with ALL (25%). Both partner genes are important for the development and maintenance of normal haematopoiesis. The TEL part contains domains interacting with the mSin3, N-CoR and HDAC-3 corepressors. A part of the AML1 gene involved in the fusion carries a DNA binding domain. TEL/AML1 protein seems to behave like a repressor blocking the expression of the genes originally transactivated by AML1. In our previous experiments we monitored the changes of immunophenotype after histone deacetylase inhibitors (HDACi; Valproate(VPA), Trichostatin A(TSA)) administration of TEL/AML1[+] cells. The expression of differential markers (RAG1, TdT) was confirmed on RNA level (RAG1-control(C)/TSA $p<0.0001$, C/VPA $p=0.0002$; TdT - C/TSA $p<0.0001$, C/VPA $p=0.01$). We have analysed our and publicly available results of ALL expression profiling and have chosen 5 genes specific for TEL/AML1 patients and influenced by HDACi administration. Expression of JunD, ACK1, PAK1 and PDGFRB in TEL/AML1[+] patients as well as in the tested cells was downregulated with an increased expression after VPA/TSA treatment. TCF4 gene was upregulated and the effect of HDACi was its contrary. We confirmed changes of expression levels by RQ-RT-PCR: JunD – C/TSA $p=0.013$, C/VPA $p=0.0008$; PDGFRB – C/TSA $p< 0.0001$ C/VPA $p=0.016$; TCF4 – C/ TSA $p< 0.0001$ C/VPA $p=0.0002$; ACK1 – C/VPA $p=0.07$. These genes are fundamental for cell cycle progression and proliferation therefore their role in leukaemogenesis is presumed. These data support the hypothesis that the effect of HDACi on TEL/AML1[+] cells is directly related to the function of TEL/AML1 protein, and the treatment with HDACi is able to release cells from differentiation block caused by TEL/AML1 aberrant transcription factor.

Grant support: 75/2005(GAUK), 301/D0189(GAČR), #8136(IGA MZ), MSM0021620813.

14:45

Identification of two potential molecular determinants of an attenuated temperature-sensitive phenotype of tick-borne encephalitis virus

DANIEL RŮŽEK, KOPECKÝ J, ŠTĚRBA J, GOLOVCHENKO M, RUDENKO N, GRUBHOFFER L

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Tick-borne encephalitis virus is an important arbovirus causing about 3000 reported human cases in Europe annually. An attenuated temperature-sensitive strain of tick-borne encephalitis virus, named ts263, was isolated from the tick *Ixodes ricinus*. This virus was non-virulent for adult mice after subcutaneous inoculation. Plaques produced by this virus were small and sometimes barely discernible. The temperature-sensitive phenotype was characterized by the lower ability of the virus to replicate at the nonpermissive temperature (40 °C) – the titre of the virus was lower by about three orders of magnitude. Surprisingly, a virulent temperature-resistant variant was identified in the quasispecies of the attenuated strain. This variant showed high degree of neuroinvasiveness and formed large plaques in cell culture. The pathogenesis of infections caused by the parental strain and the temperature-resistant variant was completely different. After the s.c. inoculation of laboratory mice with the parental strain ts263, the virus was detected in all studied lymph nodes as well as in the blood, spleen and brain, Otherwise, the s.c. inoculation with the temperature-resistant variant of ts263 strain lead to efficient replication of the virus in all studied organs. The complete genomic sequence of the temperature-resistant variant was determined and compared with the previously published sequence of the parental strain. The comparison revealed two differences in the deduced amino acid sequence of nonstructural proteins NS2B and NS3, and 218 nucleotides long deletion within the variable segment of the 3′ NCR, but with no effect on the character of the 3′ conserved region. In conclusion, the two amino acid substitutions are potential molecular determinants of the attenuated temperature-sensitive phenotype of the strain 263.

(Supported by the grants MSM 6007665801, GACR 524/031336, and SGA2005 (Faculty of biological sciences, University of South Bohemia)

15:00

Male gametophyte development: a transcriptomic view

HONYS D[1,2], REŇÁK D[1,3], DUPĽÁKOVÁ N[1,2], SZE H[4], TWELL D[5]

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The haploid male gametophyte generation of flowering plants consists of two or three celled pollen grains. The highly reduced cell lineage and functional specialization of the male gametophyte are thought to be key factors in the reproductive fitness and evolutionary success of flowering plants. Pollen ontogeny also provides an attractive model of cellular development in which to dissect the cellular networks that control cell growth, asymmetric cell division, cellular differentiation and intercellular communication. The progression from proliferating microspores to terminally differentiated pollen is characterised by large-scale repression of early programme genes and the activation of a unique late gene expression programe in maturing pollen. These data provide a quantum increase in knowledge concerning gametophytic transcription and lay the foundations for new genomic-led studies of the regulatory networks and cellular functions that operate to specify male gametophyte development.
Authors gratefully acknowledge the financial support through grants B6038409 (GA AV ČR), 1K03018 (MŠMT) and Royal Society Joint Project Grant.

15:45

RNAi-based functional study of RNA editing in kinetoplastids

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RNA editing in kinetoplastid protozoa transforms mitochondrial pre-mRNAs into functional mRNAs by the precise insertion and deletion of uridine residues as specified by small guide (g) RNAs. It is achieved by a coordinated series of enzymatic steps that cut the pre-mRNA, add/remove uridines, and ligate the mRNA. The process is performed by the editosome, a macromolecular complex of at least 20 proteins with widely different functions, and additional proteins that are not part of the editosome. Functional analysis of proteins essential for proper RNA editing, most of which are present in the mitochondrion in miniscule amounts, is amenable only via methods of reverse genetics, in particular RNA interference. Our experience with RNA interference in *Trypanosoma brucei* as well as our current understanding of RNA editing will be discussed.

16:15

Silencing of ETV6/RUNX1 fusion mRNA using siRNA

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The presence of chimeric gene ETV6/RUNX1 (TEL/AML1) is the single most frequent non-random genetic aberration in childhood ALL occurring in 20-25% of patients. Despite this high frequency, the molecular mechanism of the role of ETV6/RUNX1 protein in leukaemic transformation is still unclear. Both partner genes are important for the development and maintenance of normal hematopoiesis. RNA interference (RNAi) is a powerful tool for investigation of the role of fusion genes in malignancies. To our knowledge, no RNAi system for ETV6/RUNX1 has been reported so far, in contrast to other fusion genes in leukemias. To enable studying of the role of ETV6/RUNX1 fusion protein, we established ETV6/RUNX1 mRNA silencing system. Designer programs fail to find siRNA (small interfering RNA) oligonucleotides that would fulfill general criteria and concurrently spanning ETV6/RUNX1 fusion region. Therefore, we designed a series of 11 siRNA oligonucleotides (25-based length) spanning the fusion. Oligonucleotides were specific for ETV6/RUNX1 only and didn`t affect wild ETV6 or RUNX1 genes. The efficiency of all particular oligonucleotides was tested on bicistronic construct where ETV6/RUNX1 gene was cloned with IRES-GFP (CMV-ETV6/RUNX1-IRES-GFP). This model allows high-throughput, simple and rapid assessment of siRNA efficacy by flow cytometry as a decrease of GFP fluorescence, moreover, overcomes troublesome and low efficient transfection of lymphoblastic cells. We measured the knock-down effect as a decrease of GFP fluorescence intensity after 24 and 48 hours after transfection with siRNA oligonucleotides. We detected a decrease 35 - 68% of GFP fluorescence after 24 hours and 49-81% after 48 hours after the treatment with ETV6/RUNX1 siRNAs in comparison to CMV-ETV6/RUNX1-IRES-GFP transfected cells with control siRNA. This system allowed us to select the most efficient oligonucleotide for the subsequent study of knock-down of ETV6/RUNX1 fusion gene in genuine ETV6/RUNX1-positive cells.

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16:30

In Vitro suppression of inducible NO synthase expression using siRNA

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Nitric oxide (NO), as a potent gaseous molecule serves many physiologic roles within an organism. With respect to innate immunity, a large amount of NO can be produced by macrophages as a toxic effector molecule in response to infection. However in some cases overproduction of NO can lead to increased inflammation and subsequent detrimental pathophysiology. The production of NO by infiltrating macrophages can cause damage to tissue grafts following transplantation. NO production by macrophages is dependent on the inducible NO synthase enzyme (iNOS) thus limiting iNOS production was the object of this study. Using mouse macrophage cell line P388D1 we inhibited iNOS expression with siRNA. *In vitro*, liposome based siRNA transfection resulted in specific iNOS silencing at the mRNA and protein level as measured by Real time RT-PCR and Western Blot, respectively. IL-6 production by P388D1 cells was not influenced, indicating a specific siRNA effect. Furthermore iNOS suppression strongly depleted the levels of NO in the cell culture medium. This study showed that RNAi technology can be applied for specific iNOS inhibition *in vitro* and could potentially be used to treat conditions where NO is over-produced as a result of increased iNOS activity.

16:45

Arrays – non-invasively directly from the skin

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Introduction: Microarray techniques permit the quantification of gene expression. This method allows to monitor changes in gene expression of selected markers after treatment with tested raw materials. The advantage of obtaining biological material with strips (D-squame strips, CuDerm Corp., USA) relies on the non-invasivness of the procedure. Cells are obtained through stripping from the upper layers of epidermis and are then submitted to RNA isolation (Trizol Reagent); gaining hundreds of ng RNA.
Methods: After RNA isolation, a double-round of RNA amplification was performed acccording to Eberwine protocol. Labelled and amplified RNA were then hybridized through the use of chips placed on the bottom of Array microtubes (Clondiag, chip technologies, GmbH, Jena, Germany), and spots were analyzed with IconoClust software. Normalization and graph analyses were carried out using Microsoft Excel and Origin.
Results: Tamarind, the antioxidant from seed coats of the asiatic plant Tamarindus indica, showed photoprotective effects after UVB irradiation. Its ability to atennuate the appearance of erythem was confirmed on a transcriptional level. Tamarind causes down-regulation of proinflammatory factors.
Lignified material, which is extracted from sawdust, was the next antioxidant to be studied. The transcriptional profiling of lignin points out to its ability to increase sphingosine production; thereby, offering protection against microbial and fungal agents. Futhermore, it may supress phospholipase A2 and lipoxygenase E levels. These proinflammatory markers are significantly enhanced in skin diseases like psoriasis and ichthyosiform erythroderma.
Microarray experiments involving arabinogalactan and hyaluronan (very low molecular weight) did not show such clear results after examining of the cutaneous desquamation. The existence of an anormous biological variability in responses was found for the two substances.

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17:00

Arabidopsis Gene Family Profiler – a new easy-to-use family-oriented gene expression database

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The rapidly increasing volume of publicly available gene expression datasets for Arabidopsis now demands an environment suitable for easy orientation and that enables genome-targeted questions about expression patterns to be answered. We present a currated gene family-oriented gene expression database with a user-friendly graphic interface. Arabidopsis Gene Family Profiler (GFP) gives the user access to normalised Affymetrix ATH1 microarray data collected from NASC within the scope of the AffyWatch Service (Craigon et al. 2004). The database contains transcriptomic data for number of tissues at various developmental stages from wild type plants gathered from nearly 350 gene chips.
The Arabidopsis GFP database has been designed as an easy-to-use tool for users needing an easily accessible resource for expression data of either single genes, pre-defined gene families or custom user-defined gene sets, with the further possibility of keyword search. The environment enables users to access individual chip experiments and mean data for all appropriate microarrays. Arabidopsis Gene Family Profiler presents a user-friendly web interface using both graphic and text output. Data are stored at the MySQL server and individual queries are created in PHP script. The distinguishing features of Arabidopsis Gene Family Profiler database are 1) presentation of normalised datasets (Affymetrix MAS5 algorithm and calculation of model-based gene-expression values based on the Perfect Match-only model); 2) an intuitive interface; 3) an interactive "virtual plant" visualising the spatial and developmental expression profiles of both gene families and individual genes. Altogether Arabidopsis GFP gives users the possibility to start with simple global questions that can be further refined as highly targeted ones.
We gratefully acknowledge support from the GA ASCR (Grant B6038409).

Craigon DJ, James N, Okyere J, Higgins J, Jotham J & May S (2004) Nucleic Acids Res. 32, D575-D577.

17:15

The analysis of plant mitochondrial transcripts by means of non-radioactive Northern hybridisation and quantitative RT PCR

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This contribution deals with the transcription analysis of plant mitochondrial chimaeric genes. Methods based on reverse transcription followed by PCR (RT PCR) have been routinely applied for general gene expression analyses in the course of the last decade. They are sensitive and simple. Quantitative RT PCR (qRT PCR) using real-time PCR enables accurate quantification of mRNA abundance at the level of a single cell, which is far beyond the sensitivity range of Northerns. However, Northern hybridisation cannot be simply replaced by RT PCR in any experimental design. It is for example unavoidable in analyses trying to find the number and size of mRNAs transcribed from the same gene and spliced alternatively. In most studies of plant gene expression, Northerns with ^{32}P labelled probe are utilized. Because ^{32}P produces radioactive waste, alternatives are preferable. To adopt non-radiocative Northern procedure, we optimized the method of digoxigenin (DIG) labelling of probe to study transcription of mitochondrial *AtpA* gene in *Silene vulgaris.*

We used PCR labelling kit (Roche) to label 1.4 kb PCR fragment derived from mitochondrial AtpA gene. We compared membranes prepared by classical blotting procedure from either MOPS/formaldehyde agarose gels, or from TAE agarose gels prepared according to Mašek et al. (2005). The both electrophoretic methods brought information about the number, size and abundance of *AtpA* transcripts. The modification of MOPS/formaldehyde electrophoresis in 2% agarose gel with lower concentration of formaldehyde produced the sharpest and the best separated bands.

We designed primers that generated 200bp PCR fragments for different regions of the *Atp1* gene in plant mitochondria. qRT PCR was preformed with randomly primed cDNA to ensure reverse transcription of non- polyA tail mitochondrial transcripts. Three different levels of transcription were detected depending on the portion of *AtpA* gene used for amplification. These results are consistent with a chimaeric strutcure of some transcripts. Further experiments using respective PCR fragments as DIG labelled probe in non-radioactive Northerns are in progress.

Literature

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POSTERS

(in alphabetical order)

New tools for the manipulation of microspore gene expression

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Ongoing characterisation of male gametophyte gene functions requires the introduction of tools enabling spatially and temporally targeted expression of both marker and modified genes of interest. Compared to the limited current resources, promoters driving the expression at earlier developmental stages are required. Available transcriptomic datasets covering four stages of male gametophyte development in Arabidopsis (Honys and Twell 2004) were used to identify candidates showing microspore-specific gene expression. The putative specific expression profiles were verified by RT-PCR analysis. Verified candidate promoters were cloned into pKGWFS7 vectors (GATEWAY) and their specificity was tested by in situ GUS detection in transformed plants. Two promoters (MSP1, MSP2) showing early expression profiles were found to be specifically expressed in the male gametophyte and tapetum. To demonstrate their effective application, the MSP1 and MSP2 promoters were both used successfully to complement a cytokinesis defective mutant, which provides the first example of the application of microspore expression tools in Arabidopsis.

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Specific silencing of mutant antioncogene p53 by RNA interference

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Antioncogene p53 is one of the most important tumor suppressors, which after activation triggers cell cycle arrest and /or apoptosis in response to cellular stress. p53 gene is inactivated in more than half of all human cancers, either by deletion or point mutation. p53 is biologically active as a tetramer, and therefore the mutant protein subunit can acts as dominant negative inhibitor of wt p53. RNA interference may be used to suppress the point-mutated p53 gene and restore wt p53 function.
Gene silencing trough RNA interference (RNAi) triggered by double stranded RNA molecules is recognized as a mechanism of cellular protection against molecular parasites such as viruses and transposons and as a mechanism for post-transcriptional gene silencing (PTGS). The strict dependence of specific siRNA on the complete sequence complementarity to target mRNA allows selective recognition and degradation of the mutant version of mRNA, that differs from the wild-type version of mRNA by as few as a single nucleotide.
We designed vector pPRO.sh.RNA, (i) with constitutive expression of shRNA sequences targeted against mutant p53, (ii) green fluorescent protein (EGFP) to detect transfection efficiency and enable cell sorting (FACS), (iii) pUC19 origin of replication and kanamycin resistance in E.coli and (iv) neomycin-resistance cassette.
We have successfully silenced expression of mutant variant of p53 protein in cell lines derived from Ewing´s sarcoma. Silencing of the mutant (Arg273His) p53 in STA-ET-7.2 cell line was more than four fold higher in comparison to silencing of wt p53 in TC252 cell line.
Specific silencing of mutant p53 gene provides a powerful tool for functional genomics and potential approach for anticancer therapy.

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Direct reverse transcription efficiency measurement for real-time RT-PCR normalization

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Real-time RT-PCR (qRT-PCR) is typically used to assay transcript abundance by measuring a specific cDNA level. It is very sensitive and covers an enormous dynamic range of cDNA concentrations but it depends on reliable normalization. Recently, the prefered method of qRT-PCR normalization takes advantage of reference genes with expression believed to be stable as an internal control. This corrects the measured transcript levels for variable starting RNA amount and also for differences in the reverse transcription efficiency. This second point is very important as it was shown that RT is a major source of real-time RT-PCR variation (Stahlberg et al. 2004). Application of reference genes suffers from the fact that no gene showing completely invariant expression exists. Therefore, it is necessary to select and carefully check appropriate reference genes to be used in specific treatment or experimental conditions.

Another approach utilizes the input RNA amount (obtained either from spectrofotometric (A260) or fluorometric (RiboGreen) quantification) as a correction factor for each sample. It is not influenced by fluctuations in reference gene expression but the method strongly depends on the reproducibility of the RT reaction and requires invariant proportion of mRNA in the total RNA sample.

We present an alternative approach to qPCR normalization that employs the amount of total cDNA pool generated during the RT for the correction. The amount of cDNA reflects the variability in the starting material as well as any differences in the RT efficiency. For the detection, we tested 3 fluorescent dyes: Pico Green, SYBR GreenI and Ribo Green. Ribo Green proved to be the most suitable of the three. As none of the fluorescent dyes discriminates enough between RNA and DNA, we had to find a way how to reproducibly remove RNA, which otherwise produces most fluorescence signal.

Stahlberg et al.(2004), Clin Chem. 2004 Mar;50(3):509-15

Hepatitis C virus IRES is functional in yeast cells

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Chronic complications of HCV infection have become a serious health problem in the last decade. Integrity of 5′-noncoding region (NCR) containing also the internal ribosome entry site (IRES) is essential for an efficient synthesis of the viral polyprotein and thus for the HCV propagation in mammalian cells. Up to date, HCV IRES demonstrates one of the best-studied viral IRESes. Although, there is no extra need for the presence of canonical initiation factors, it is believed that function of HCV IRES is restricted only to mammalian cells.

For testing of the HCV IRES activity we developed a specialised and sensitive system based on the bicistronic vectors and engineered yeast strains, which allows an enhancement of the measured signal by in vivo coupled transcription and enzymatic detection. To exclude the possibility of the presence of cryptic promoters and other experimental artefacts we prepared a set of control vectors as well as vectors with HCV IRES sequences containing various point mutations. Our data suggest that HCV IRES is functional in yeast cells and a molecular mode of its function remains largely unchanged. We believe that this novel reporter system can be used both for basic research focused on cellular and viral IRESes and as a fast and less expensive approach for screening of antiviral drugs targeted against HCV IRES.

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IRESite - The database of experimentally studied viral and cellular IRES elements

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This project focuses on the IRES elements (Internal Ribosome Entry Site) which play important role in the initiation of translation in eukaryotes. IRES elements were originally found in eukaryotic viruses and nowadays are known in over 50 distinct viruses including human and animal pathogens. IRES elements were also discovered as regulatory elements in 5' UTRs of over 70 eukaryotic cellular mRNA molecules. However, it is not yet clear whether the cellular IRES elements are more widespread.

We present a database solution containing first while still small subset of the vast amount of data currently available in the literature. The IRESite database records 92 biologically relevant aspects of every experiment, for example nature of IRES element, its functionality/defectivity, its origin, size, sequence, structure, relative position in respect to surrounding protein coding regions, positive/negative controls used, reporter genes used to monitor IRES activity, measured reporter protein yields/activities and references to original publications as well as cross-reference to other databases and comments from submitters and curators. Further presented are known similarities to rRNA sequence, RNA-protein interactions and special care is given to annotation of promoter-like regions. The annotated data are bound to complete, full-lenght mRNA and even accompanied by original plasmid vector sequences (all hand-crafted according to original publications).

New data can be submitted through the publicly available curated web-based interface at http://www.iresite.org. We continue to fill the database with more data and would like to invite scientists to kindly contribute their results.

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Role of transcription factors in early male gametophyte development of Arabidopsis thaliana

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The haploid male gametophyte generation of flowering plants consists of two or three celled pollen grains and pollen tubes as an evolutionary result of structural and functional reductions but performs a vital role in the plant life cycle. Hence pollen ontogeny provides an attractive model for the study of fundamental developmental processes like cell growth and division, cellular differentiation and intercellular communication. Despite a long-term research on the field of plant sexual reproduction, the developmental changes and regulatory mechanisms during pollen grain formation have not been properly described yet.

Our research aims to characterize the role of transcription factors (TF) in male gametophyte development of Arabidopsis thaliana with special focus on early developmental stages such as uninucleate microspores and bicellular pollen grains. Exploiting microarray technologies (Honys and Twell, 2003, 2004) followed by careful bioinformatic analyses, we selected about 30 genes encoding putative TFs expressed specifically during the developmental stage considered. To prove the importance of selected TFs in development, we analysed respective T-DNA insertion lines. After PCR verification of all insertions, we performed the phenotype screening for aborted or structurally abnormal pollen grains by both light and UV microscopy. The second approach comprises the search for segregation ratio distortion demonstrating the functional significance of examined gene without visible phenotype. Selected positive mutant lines will be genetically characterised to reveal the transmissibility as well as the recessive or dominant character of the mutation. Moreover, the influence of TF genes on downstream regulation will be examined by comparative transcriptome analysis of both TF mutant lines and wild type plants in order to determine their role in gametophytic gene regulatory networks.

Acknowledgment
We gratefully acknowledge support from the GA ASCR (Grant B6038409).

Honys D., Twell D. (2003) Plant Physiol. 132, 640-52
Honys D., Twell D. (2004) Genome Biol. 5: R85

Construction of a new bicistronic reporter assay system for analysis of the diversity of HCV IRES quasispecies

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Hepatitis C came out from the pool of unidentified, non-A, non-B hepatitis in 1989 year and shortly after this description has become recognised as one of major health problems of the present time. It has been estimated that at least 20 to 30 % of patients chronically infected with hepatitis C virus (HCV) will develop a liver cirrhosis and subsequently some of them a liver carcinoma. Serious problem in a HCV research and in a search for new antiviral therapy is the absence of the satisfactory animal model, of course with the exception of chimpanzee. Thus, most of the current knowledge has been obtained from studies of clinical isolates or by deduction from data obtained in experiments with transient expression of coding or non-coding regions of the viral genome in the various human cell lines.

The major objective of our work is the study of diversity of HCV quasispecies evolving frequently in an every single patient. We focus our research on developing of suitable system for measuring of the HCV IRES activities simultaneously both on the level of the single individual IRES sequence and on the level covering the whole population of the IRES quasispecies existing in a clinical sample at the given time. We prepared and present here a novel bicistronic reporter vector pRG. Due to the organization of this bicistronic vector we are able to analyse fluorescence of thousands of particular cells in a short time using flow cytometry.

For the construction of the novel vector we used Discosoma sp. red fluorescent protein (DsRed2) and enhanced green fluorescent protein (EGFP) from Aequoria victoria as a first and second reporter gene respectively. We prepared several control vectors as well as testing vectors containing wild type HCV IRES or the library of randomly mutated HCV IRESes inserted between the two reporter genes.

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Identification of flax miRNAs by cDNA cloning - project proposal

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TARGETED HORIZONS AND ESSENTIAL PHILOSOPHY:

Better understanding of molecular processes controlling flax vascular fibre formation, which are probably strongly governed by miRNA part of flax transcriptom, should bring about quite new knowledge on gene expression network in a non-model plant, fibre crop Linum usitatissimum L (flax). Description of flax miRNA pool may help to set up the toolbox for upcoming novel biotechnologies aimed to targeted manipulation with flax fibre tissue in order to modify its quality for either classical textile applications or modern non-textile industrial applications walking to design and production of new biodegradable "green" composite plastics (Wong et al. 2004).

We arranged and submitted for granting the project proposal, which is aimed to the first step of the way toward understanding of miRNA regulatory functioning in fibre vascular tissues, i.e. to the isolation and sequence characterisation of fibre miRNAs.

AIMS OF THE PROJECT:

We plan:

a) to perform introductory characterization of miRNA pool of flax transcriptome on highly sensitive modifications of Northern blots using heterologous probes

b) to isolate miRNAs from flax fibres by cloning following the approach of Lau et al. (2001) with recent modifications of Ambros (2005)

c) to characterize isolated and cloned miRNAs by sequencing

d) to look at the phylogeny context of obtained sequences by comparison to already described miRNAs, of plant as well as of Metazoan origin

e) to verify presence of newly isolated and cloned flax-fibre miRNAs in total RNA from fibre tissue by highly sensitive Northern blots and by extremely sensitive in-solution hybridization.

Ambros V. (2005),
http://banjo.dartmouth.edu/lab/MicroRNAs/Ambros_microRNAcloning.htm
Lau, et al. (2001), Science 294: 858.
Wong, et al. (2004), Macromol.Mater.Eng., 289: 447

Gene expression analysis in cold and drought treated barley (*Hordeum vulgare* L.) using real-time quantitative reverse transcription PCR

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The study was aiming to investigate the differences in expression of selected genes using a real-time quantitative reverse transcription PCR (qRT-PCR) in barleys stressed by cold and drought. The tested *Dhn* genes used belong to the multi-gene family encoding dehydrins in barley. Dehydrins are involved in plant response to cold and drought stresses. *Dhn* genes expression in genotypes grown in precisely defined stress condition was studied. Total RNAs were extracted from spring cultivar Atlas68 and winter cultivar Igri after 0, 0.5, 1, 2, 3, 7 and 14 days of the cold and drought stress application. The methodology of qRT-PCR in a two-step procedure including samples preparation is described here.

Key words: Dehydrin · Cold Stress · Drought Stress · Gene Expression · Barley · Real-Time qRT-PCR · RNA

Poděkování za podporu