Instituto Juan March
de Estudios e Investigaciones

50 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

From Transcript to Protein: mRNA Processing, Transport and Translation

Organized by

I. W. Mattaj, J. Ortín and J. Valcárcel

M. Bach-Elias
J. G. Belasco
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INTRODUCTION

Juan Ortín

Biological research very frequently involves reductionist experimental approaches on very specialized aspects of the cell structure, regulation or fate. However, it is becoming more evident every day that biological phenomena are profoundly and extensively interrelated and interdependent. For a virologist like me, an outsider in the Cell Biology field, it was quite a surprise when work of our group, as well as others, showed that the influenza virus NS1 protein, a small non-structural protein, was able to alter several aspects of the gene expression program of the cell, including pre-mRNA splicing, nucleocytoplasmic transport and mRNA translation. Therefore, it was not a fully altruistic idea to propose to the Centre of International Meetings on Biology ("Instituto Juan March de Estudios e Investigaciones") a Workshop to integrate discussions on the post-transcriptional steps involved in gene expression. Looking at the state of the field, I felt that it was particularly appropriate to look at the recent advances with an integrative perspective and that the Spanish scientists, myself included, could learn very much from it.

The organization of such a meeting would have been impossible without the active role of relevant researchers in the field. I was very lucky to have Iain Mattaj and Juan Valcarcel as co-organizers, who helped very much in the difficult task of selecting, among the many excellent scientists covering these topics, those that would make an appropriate blend for a successful meeting.

It was the purpose of the meeting to find interconnections between the several steps that take place from the point a primary transcript is synthesized up to the moment its corresponding mRNA is actively translated and, eventually, degraded. After three days of intensive exchange of ideas, the outcome of the workshop exceeded our expectations. Indeed, both the oral and poster presentations, as well as the very active discussions, supported previously suspected connections and opened our minds to unexpected new ones.

The meeting took place in the incomparable atmosphere of the Fundacion Juan March premises, surrounded by art exhibitions and concerts, and much of its success should be attributed to the expert and elegant dedication of Andres Gonzalez and his co-workers, who provided us with every facility one could think of to allow our work to proceed smoothly. On behalf of everyone in the meeting, I should like to transmit our gratitude to them.

FIRST SESSION

Chairperson: Walter Keller

EARLY STEPS OF THE SPLICEOSOME ASSEMBLY: IMPORTANCE OF BRANCHPOINT REGION RECOGNITION

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5' splice site and branchpoint intronic conserved regions are important for spliceosome assembly through base pairing with U1 and U2 snRNAs, respectively. Commitment complexes formed in vitro involve the 5' splice site and the branchpoint region. They contain the U1 snRNP particle and associated proteins but do not require U2 base pairing with pre-mRNA. This result suggests that initial steps of spliceosome formation involves protein:RNA interactions that bring together intronic sequences involved in the first catalytic splicing step. In vivo, splicing defect analyses do not discriminate between spliceosome formation or splicing catalysis defects and the precise contribution of the conserved nucleotides for either process remains unclear. To identify sequence requirements for spliceosome formation in vivo, we analyzed many intronic point mutations with a previously described pre-mRNA export assay[1] in which a synthetic open reading frame intron is inserted in a hybrid *CYC1-LacZ* gene under the control of a galactose inducible promotor. Measurement of ß-galactosidase activity, after galactose induction, is used as an indication of pre-mRNA export. The nuclear escape of pre-mRNA is a mark of an intron recognition defect.

Several point mutations were made at specific positions at the 5' splice site: intronic mutations exhibit a strong pre-mRNA export phenotype whereas exonic mutations have no phenotype. This confirms that this assay points toward positions involved in intron recognition. Then we looked for the function of branchpoint region nucleotides by making a random mutant library of the 12-nucleotide sequence TCTATACTAACA. We screened 900 yeast transformants using a qualitative filter assay for ß-galactosidase activity after galactose induction. All 85 positive candidates - at least a 2 fold increase of the wildtype activity - were further analyzed and sequenced. We identified 39 point mutants: all but one (see below) are located within the TACTAAC sequence at any of the seven positions and exhibit a more or less severe export phenotype. Any double mutant involves at least one mutation in the TACTAAC sequence. This result shows that the recognition of the intron and/or the stability of spliceosome involve mainly the whole TACTAAC sequence.

Analysis of pre-mRNA export and splicing deficiency was made for every single substitution in the TACTAAC sequence. Splicing efficiencies were analyzed by primer extension and by sub-cloning mutant introns in a reporter gene whose ß-galactosidase activity reflects splicing. One of the striking results is that the amount of the exported pre-mRNA does not correlate directly with the intensity of the splicing defect. For example, at some positions, the three substitutions have very different effects on splicing, while they exhibit similar pre-mRNA export phenotypes. Whereas splicing phenotypes of various mutants can be explained by different U2 base pairing efficiency, pre-mRNA export could not. This result suggests the presence of other factors interacting with the branchpoint region.

One export mutant was the A to C substitution at the position preceding the TACTAAC sequence. An analysis of the four substitutions at this position shows that a T nucleotide allows an almost undetectable amount of pre-mRNA

export, whereas C has the strongest pre-mRNA export phenotype. Contrary to mutants in the TACTAAC sequence, we find for these mutants a very good correlation between pre-mRNA export and splicing deficiency. This result suggests that the position preceding the TACTAAC sequence might be an important parameter for intron recognition rather than for splicing catalysis. This hierarchy between the four nucleotides at the position preceding the TACTAAC sequence was also found among natural introns whose sequences exhibit a strong bias toward a T and a rare occurrence of a C residue.

Several trans-acting factors, Prp9p, 11p, 21p and Mud2p, were analyzed for their involvement in the pre-mRNA export phenotype of branchpoint region mutants. Prp9p, 11p and 21p are implicated in the U2 snRNP binding to the pre-mRNA and Mud2p is a U1 snRNP-associated protein known to be important for the formation of commitment complexes in vitro. Overexpression of *PRP9*, *11* or *21* genes do not modify the pre-mRNA export phenotype of branchpoint region mutants. On the contrary, the expression of the *MUD2* gene modulates the phenotype of mutants at the position preceding the TACTAAC: a strain disrupted for the *MUD2* gene exhibits an increased pre-mRNA export compared to a wildtype strain and this effect is specific for this mutant position.

In conclusion, the recognition of an intron involves the complete TACTAAC sequence and the preceding position. This study emphasizes the importance of this conserved intronic sequence, brought together with the 5' splice site, as an essential element of intron recognition. Binding of splicing factors occur at both sites but stable formation of commitment complexes requires joint recognition through protein factors, among them the Mud2p protein.

1 Cell (1989), 57, 573-583.

STRUCTURE AND FUNCTION OF SMALL NUCLEAR RIBONUCLEOPROTEINS FROM *SACCHAROMYCES CEREVISIAE*

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Small Nuclear RiboNucleoProteins (snRNPs) are particles found in the nucleus of eukaryotic cells that are composed of one RNA associated with one or more proteins. The spliceosomal U1, U2, U4 and U5 snRNAs are associated with Sm proteins as well as snRNP-specific protein(s). The eight small Sm proteins form a heteromeric RNA binding complex that plays a major role in snRNP biogenesis. They are also a major target for autoantibodies in the human disease systemic lupus erythematosus. By sequence comparison we have shown that all the known Sm proteins share a common sequence motif suggesting that they have similar fold. This might explain their immunological cross-reactivity. Database searches uncovered a large number of Sm-like proteins from plants, animals and fungi. Some of the divergent yeast proteins were shown to be true Sm proteins as they associate with the yeast U1, U2, U4/U6 and U5 snRNAs. Surprisingly, one Sm-like protein was found to be a component of the U6 snRNP (1). These findings have implications for the structure of the Sm protein complex, spliceosomal snRNP evolution, snRNA transport, snRNA modification and the involvement of Sm proteins in systemic lupus erythematosus. Beside the Sm proteins, we have also characterised a protein of the RNase P and MRP snRNPs which are involved in tRNA and rRNA processing respectively (2).

We are also analysing the function of these various snRNPs. We have developed an in vitro assay for RNase MRP which allowed the identification of the nucleolar RNA cleaved by this enzyme. We are also interested in the role of snRNP in pre-mRNA splicing. We have shown that repetitive recognition of 5' splice site sequences through base-pairing with the U1, U5 and U6 snRNAs is required for splicing accuracy

(3). Further work is in progress to understand the role of snRNPs in splice site selection and catalysis during RNA processing.

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NATIVE-CAPTURE ELUTION OF SPLICEOSOMES

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A new approach is taken to purify native spliceosomes. It is based on the use of some BrU or BrdUTP residues that have been incorporated at the 3' end of the pre-mRNA or in some internal U residues of the pre-mRNA. Having in mind that antibodies anti-cap have been shown to be an important tool to select spliceosomal ribonucleoproteins, in the same way antibodies anti-BrU or anti-BrdU could be also useful to capture spliceosomes. Experiments done with comercial and monoclonal anti-BrdU have shown that at least 20% of total spliceosomes could be selected with pre-mRNA elongated with BrdUTP and that they can be eluted by competition with free BrdUTP. These results, animate to prepare polyclonal rabbit/ascites antibodies in order to selectively purify antibodies anti-BrU that compite with free BrU, that could be more free of RNAses. In parallel, to give validity to the described method, a complementation assay was developped. In this assay a HeLa splicing extract that performs the second splicing reaction in a very slow way was complemented with purified yeast UsnRNPs. These yeast UsnRNPs stimulate the second HeLa splicing reaction, that confirms once more the conservation of splicing factors from yeast to HeLa. Experiments are ongoing to combine the two proposed systems, in order to study whether the BrU-captured spliceosomes are native enough to be complemented with second reaction splicing factors.

A Novel Spliceosome Containing U11, U12 and U5 snRNPs Excises a Minor Class (AT-AC) Intron in vitro

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A minor class of introns with noncanonical splice (AT-AC) and branch site sequences exists in metazoan protein coding genes(1). We have established a HeLa cell in vitro system that accurately splices a pre-mRNA substrate containing such an intron from the human P120 gene. Splicing occurs via a lariat intermediate whose branch site A residue is predicted to bulge from a duplex formed with the low abundance U12 snRNP(2), which we confirm by psoralen crosslinking. Native gel electrophoresis reveals that U11, U12 and U5 snRNPs assemble onto the P120 pre-mRNA to form splicing complexes that resemble the A, B and C complexes characterized for the major spliceosome. Inhibition of P120 splicing by 2'-O-methyl oligonucleotides complementary to U12 or U5 demonstrates that U12 and U5 snRNPs perform essential roles in the AT-AC spliceosome. Our results suggest the existence of lower abundance U4 and U6 snRNP analogs, and raise many questions concerning the AT-AC spliceosome.

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SECOND SESSION

Chairperson: Joan A. Steitz

ANALYSIS OF CONSTITUTIVE AND ALTERNATIVE mRNA SPLICING FACTORS

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We use biochemical and molecular approaches to identify and characterize cellular components required for splicing and/or involved in regulating alternative splicing in human cells. We recently identified a 343-amino acid human protein, hPRP18, which has 30% identity to the *S. cerevisiae* splicing factor PRP18. The homology is primarily in the C-terminal third of the protein, which is also conserved in rice and nematode ESTs. We expressed hPRP18 in *E. coli* and raised antibodies to it, which were used to deplete hPRP18 from HeLa cell nuclear extracts. Immunodepletion specifically and completely blocks the second step of splicing, leading to accumulation of splicing intermediates. Recombinant hPRP18 restores the second step of splicing, demonstrating that hPRP18 is essential for catalytic step II. Recombinant yeast PRP18 also restores activity to the depleted human extract, showing that the yeast and human proteins are functional homologs. However, hPRP18 cannot functionally replace yeast PRP18, either *in vivo* or *in vitro*. hPRP18 does not appear to be stably associated with snRNPs. Unexpectedly, anti-hPRP18 antibodies crossreact with a protein that appears to be a novel component of U4/U6 and U4/U6·U5 snRNP particles.

We are characterizing three families of RNA-binding proteins that act antagonistically to modulate alternative splice site selection *in vitro* or *in vivo*. The human SR protein family consists of eight known members, each of which can act independently as an essential splicing factor required for an early step in spliceosome assembly. In addition, each SR protein promotes the selection of specific alternative 5' and/or 3' splice sites in a concentration-dependent manner, and usually in a position-dependent manner. For example, the prototype SR protein, SF2/ASF, favors exon inclusion or the selection of proximal alternative 5' or 3' splice sites with a variety of pre-mRNA substrates, both *in vivo* and *in vitro*. The hnRNP A/B proteins, exemplified by hnRNP A1, antagonize SR proteins to promote distal 5' splice site use and skipping of alternative exons. We have also purified SF7A, one of several activities that antagonize SR proteins to promote distal 3' splice site selection *in vitro*. Pairwise antagonistic interactions between specific members of these three families of RNA-binding proteins may generate sufficient combinatorial diversity to account for the developmental or tissue-specific regulation of many cellular genes by alternative splicing.

To understand the structural basis for the observed substrate specificity of each SR protein in constitutive and alternative splicing, we are analyzing domain deletion and chimeric variants of SF2/ASF and other SR proteins. We found that the RS domain of SF2/ASF is not required for localization in the nucleoplasmic speckles, or for alternative splicing activity *in vivo*. Other SR proteins with a single RRM do require the RS domain for proper localization, whereas SF2/ASF has redundant localization signals in both RRMs and in the RS domain. Either RRM1 or RRM2 can be deleted without loss of alternative splicing function *in vivo*, but RRM2 appears to confer splice-site specificity with certain substrates. We also found strong substrate-specific differences between SR proteins in constitutive splicing, and are mapping both the protein domains and substrate sequences responsible for these differences.

Finally, we are continuing to study the structure and function of hnRNP A/B proteins. In the case of hnRNP A1, we found that RRM1 and RRM2, as well as the Gly-rich C-terminal domain are required for alternative splicing function. The Gly-domain is required for RNA binding, and both RRMs are necessary for sequence-specific binding. Although two RRMs are required for function, RRM duplication and swap experiments (in collaboration with S. Munroe, Marquette University) suggest that RRM2 plays a more important role. hnRNP A1, A2, and B1, all of which are active in alternative splicing, have related high-affinity binding sites, whereas hnRNP $A1^B$, which is much less active, binds non-specifically. The observed correlation between function and binding to Selex sites suggests that alternative splicing activity involves sequence-specific binding by these hnRNP proteins.

MECHANISMS OF 3' SPLICE SITE DEFINITION AND REGULATION

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Removal of introns is an essential step in the expression of genes from primary transcripts to proteins, and is often subject to regulation during cell differentiation and development. Three sequence elements mediate recognition of the 3' end of introns in higher eukaryotes: 1/ a highly conserved terminal 3' AG dinucleotide, 2/ a poorly conserved branchpoint sequence, usually located 12-35 nucleotides upstream from the AG, and 3/ a polypyrimidine (Py) tract inmediately downstream from the branchpoint. Two of these sequences participate in the chemical steps of the splicing reaction: a nucleotide in the branchpoint region forms a 2'-5' bond with the 5' end of the intron during the first step, and cleavage of the intron after the AG dinucleotide occurs during the second step.

The splicing factor U2AF^{65} binds to the Py-tract and facilitates recognition of the branchpoint by U2 snRNP. This protein has two modules: an RNA-binding domain composed of three RNP-CS motifs, and an amino-terminal arginine-serine-rich (RS) domain. We have found that when U2AF^{65} binds to the Py-tract through its RNP-CS domain, the RS region contacts the branchpoint. Mutational analysis revealed that the RS region could be substituted by heterologous RS domains present in functionally unrelated splicing factors, or even by a "synthetic" (RS)7 domain, and that the only requirement for RS domain function is the presence of positively charged aminoacids in this region of the protein. Because U2 snRNP binding involves limited and imperfect base-pairing between U2 snRNA and the branchpoint, we investigated whether the role of the positively charged RS region in contact with the branchpoint is to facilitate this otherwise unstable interaction. Our results indicate that the RS region is required for U2 snRNA-branchpoint base-pairing in nuclear extracts, and for U2AF^{65} to promote this interaction in a reconstituted system containing only the protein, U2 snRNA and pre-mRNA. Furthermore, we found a perfect correlation between the ability of the different RS mutants to support splicing, contact the branchpoint, and mediate U2 snRNA/branchpoint annealing. This targetted annealing activity of U2AF^{65} can illustrate how formation of short RNA helices, which are essential for the assembly and catalytic activation of the spliceosome, can be assisted by protein splicing factors.

The branchpoint is highly conserved in yeast and is very sensitive to mutation. We have observed that a Py-tract, which is usually absent from yeast pre-mRNAs, can suppress mutations in the branchpoint. These results suggest the existence, also in lower eukaryotes, of a U2AF^{65}-like activity able to assist U2 snRNA-branchpoint base-pairing.

Because of its central role in 3' splice site definition, U2AF^{65} is a good target candidate for regulation. Consistent with this idea, we have found that the Drosophila splicing regulator Sex-lethal (Sxl) mediates alternative 3' splice site choice in the pre-mRNA of *transformer* by antagonizing U2AF^{65} at a particular 3' splice site, which results repressed. While U2AF^{65} is a general splicing factor able to recognize multiple Py-tracts, the high selectivity of Sxl for the Py-tract of the repressed 3' splice site can explain the specificity of Sxl function. The absence from Sxl of an effector domain able to promote U2 snRNP recruitment explains why Sxl is a splicing repressor while U2AF^{65} is a splicing activator. Recent work using transgenic flies (in collaboration with Lucas Sánchez's lab, CIB-Madrid) indicates that antagonism with U2AF^{65} can explain how Sxl regulates *transformer* , but is not sufficient to explain how it regulates other target genes, suggesting additional mechanisms involved in the regulation of these other genes by Sxl.

It has been proposed that U2AF^{65} can also be the target for positive 3' splice site regulation mediated by purine-rich exon enhancers. To explore this possibility, we tested whether the presence of an exon enhancer can facilitate splicing of a pre-mRNA substrate under conditions of limited availability of U2AF^{65}. Our results suggest that the stimulation of 3' splice site usage by exon enhancers is mediated by a Py-tract-associated factor distinct from U2AF^{65}.

NUCLEAR ORGANIZATION OF SPLICING snRNPs.

Angus I. Lamond

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The small nuclear ribonucleoproteins (snRNPs) involved in pre-mRNA splicing show a complex localisation pattern in mammalian cells. They are concentrated in the nucleoplasm, excluded from nucleoli, and associate with several distinct subnuclear structures including perichromatin fibrils, clusters of interchromatin granules and coiled bodies. Probably most, if not all, splicing of pre-mRNA takes place on nascent transcripts. The maturing pre-mRNA is identified in the EM as chromatin associated fibres called perichromatin fibrils. In the light microscope transcription sites, when labeled by incorporation of Br-UTP, appear as many hundreds of bright foci widely dispersed throughout the nucleoplasm. Neither the coiled bodies, nor the clusters of interchromatin granules, are major sites of transcription, although both contain splicing snRNPs. The functions of these structures are still unknown but could include roles in snRNP assembly, disassembly, maturation, transport and/or storage.

We are interested in how subcompartments within the nucleus are assembled and how RNPs and other components are specifically targeted to these structures. As a model system for understanding RNP compartments in the nucleus we are studying the structure and assembly of coiled bodies with a view to understanding what role this conserved organelle plays in nuclear function. Coiled bodies were first described at the light microscopy level as long ago as 1903 by the famous Spanish cytologist Ramon-y-Cajal. He termed them "nucleolar accessory bodies" since they were often found in close association with the nucleolar periphery. They were later rediscovered by electron microscopists and given the name "coiled body" due to their ultrastructural resemblance to a bundle of coiled threads. More recently Tan and coworkers identified a human autoantigen that localized to coiled bodies, termed p80 coilin, and this protein has now been cloned and sequenced by several groups. Coiled bodies are found in both animal and plant cells and contain a fraction of the nucleoplasmic splicing snRNPs but not rRNA. They also contain small amounts of several nucleolar antigens.

We have undertaken a molecular analysis of p80 coilin and have shown that when the full length cDNA for coilin is epitope tagged and transiently expressed in HeLa cells the recombinant protein correctly localizes to coiled bodies. Using this transient expression assay we have studied the localization of mutant derivatives. This has shown clear evidence for a functional interaction between coiled bodies and nucleoli. Specific mutations in p80 coilin not only affect coiled bodies but also cause disruption of nucleoli, loss of RNA pol I activity and segregation of many nucleolar antigens.

I will present here data showing the phenotypes of p80 coilin mutations and discuss the implications of these data for models of nucleolar function and nuclear organisation.

The down regulation of mRNA levels by premature stop codons

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Incomplete polypeptides resulting from nonsense mutations in one allele can seriously interfere with the function of a protein e.g. by competing with the normal gene product. This is avoided by decreasing the level of the corresponding mutant mRNA. In simple organisms (e.g. yeast) this is achieved by specific degradation of the cytoplasmic mRNA. However, in higher organisms, no alteration in the half life of the cytoplasmic mutant mRNA has been observed. Accelerated decay of the nuclear mRNA or pre-mRNA induced by the premature termination of protein synthesis has been the favoured hypothesis to explain the phenomenon (reviewed by Maquat, 1995).

Antibody and T-cell receptor genes, constitute an extreme example where premature chain termination occur at high frequency. In both cases, the expressed genes involve inaccurate rearrangements of gene fragments, often resulting in out of frame rearrangements of one allele but not of the other. In addition in the case of accurately rearranged antibody genes, premature termination codons often arise by somatic hypermutation during the process of affinity maturation. As a consequence of these events the corresponding mRNA pool is depleted. However, no evidence of decay was observed with various nonsense mutants of Igκ-light chains. Instead there seemed to be accumulation of the corresponding pre-mRNA suggesting that the nonsense mutations induced an inhibition of normal splicing (Lozano, 1994).

The difficulty in the interpretation of the results obtained so far to understand how premature stop codons control mRNA levels in vertebrates, is that they rely on experiments using whole cells in tissue culture. Recently we have developed a cell free system in which inhibition of normal splicing(Aoufouchi et al 1996) correlates with in vivo down regulation of the level of mRNA harbouring premature chain termination.

It is a common assumption that the recognition of stop codons is the result of the premature termination of protein synthesis. In apparent agreement with that assumption, we will show that splicing by nuclear extracts is inhibited by in frame stop codons regardless of the neighbouring nucleotide sequence, and the mutation of initiation Met->Thr restores splicing. However, polysomes are removed during the preparation of the nuclear extracts used in the analysis of splicing. Furthermore, protein synthesis inhibitors are unable to restore splicing.

Another common assumption is that the decrease in mRNA pool is due to increase rate of decay of mRNA or pre-mRNA. We will show that both pre-

mRNA and mRNA harbouring nonsense mutations are quite stable under the conditions we analyse splicing by nuclear extracts. Furthermore, we find that the inhibition of splicing, exhibits tissue specificity since the abnormal splicing of immunoglobulin-like genes is detected with B- but not with HeLa or T-cell nuclear extracts.

These results suggests that differentiated cells contain a nuclear scanning device for the definition of the expected protein synthesis frame, before splicing takes place. However, there must be additional tissue specific factors to stop the normal splicing process when premature termination codons are detected.

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THIRD SESSION

Chairpersons: Walter Keller and Gideon Dreyfuss

Nucleo-cytoplasmic trafficking of hnRNP proteins and mRNA export

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The hnRNP proteins are the major pre-mRNA/mRNA binding proteins in eukaryotic cells and some of the most abundant proteins in the nucleus. Pre-mRNAs associate with hnRNP proteins as nascent transcripts and these proteins play important roles in the biogenesis and metabolism of mRNAs. The hnRNP proteins are localized primarily in the nucleoplasm, and several of them shuttle continuously between the nucleus and the cytoplasm. Their intracellular transport properties are therefore of considerable interest as they appear to be important for formation of mRNA and for mRNA nuclear export. We are engaged in the identification of the signals within the proteins as well as the machineries involved in these intracellular transport pathways. The hnRNP A1, a 34kD protein, is one of the most abundant hnRNP proteins and it is a nucelo-cytoplasmic shuttler. We found that a 37 amino acid domain within A1, termed M9, which bears no resemblance to classical nuclear localization signal (NLS) sequences, localizes A1 to the nucleus. M9 also contains a nuclear export signal (NES); placing M9 on proteins that are otherwise restricted to the nucleus, such as nucleoplasmin core domain (NPc), efficiently exports them to the cytoplasm. In contrast, classical NLSs cannot promote the export of NPc. The M9-mediated nuclear import and export are temperature-sensitive processes. These findings demonstrate that there is a signal-dependent, temperature-sensitive pathway for export of nuclear proteins. The M9-mediated transport pathway also operates efficiently in X. laevis oocytes. Furthermore, as M9 does not have RNA-binding activity, these results strengthen the suggestion that A1, and other shuttling hnRNP proteins, have the capacity to serve as carriers for RNA export to the cytoplasm. Candidate M9-binding proteins are being characterized. Related studies on trafficking of other RNA-binding proteins and on mRNA export will also be described.

Interactions between the HIV-1 Rev protein and nucleoporins in yeast.

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We have previously shown that the HIV-1 Rev protein promotes pre-mRNA export in the yeast S. cerevisiae. Rev's function was examined by using the CUP1 reporter gene system, which allows to monitor Rev's activity with a growth assay. Using the yeast two-hybrid system, we have identified a yeast gene the product of which interacts specifically with the wild-type Rev and Rex proteins but not with the Rev effector domain mutant M10. The protein, RIP1/NUP42, is a novel small nucleoporin, and its closest known yeast relative is a nuclear pore component also implicated in mRNA transport from nucleus to cytoplasm. Analysis of strains that overexpress RIP1 or that are deleted for the RIP1 gene show that it is important for Rev's effect on gene expression, indicating that the physical interaction is of functional significance in vivo. The results suggest that Rev directly promotes the cytoplasmic transport of suitable transcripts by targeting them to the nuclear pore. We have now extended these results in several ways. 1) We have searched for and identified several other nucleoporins, from mammals as well as yeast, that also interact with Rev in the two hybrid system; the analysis suggests that nucleoporin repeats are the Rev-interacting moieties. 2) We have analyzed the effects of 11 Rev effector domain mutants on these interactions as well as on Rev biological activity in yeast. As the activities of these mutants are well characterized in mammalian cells, their effects in yeast are instructive. 3) We are also characterizing the significance of some of these interactions, by analyzing the effect of other nucleoporin mutants on Rev function in yeast. More generally, the significance and biological role of the Rev-nucleoporin interactions will be discussed.

HIV-1 REV AS A MODEL NUCLEAR RNA EXPORT FACTOR, BRYAN R. CULLEN, Howard Hughes Medical Institute and Department of Genetics, Duke University Medical Center, Durham, NC 27710.

The HIV-1 Rev protein is the best understood protein involved in the nuclear export of specific target RNA species. Rev function requires two domains. The first is a basic domain that mediates 1) nuclear import of Rev, 2) binding of Rev to its RNA target sequence, the RRE, and 3) multimerization of Rev on the target RNA. A second critical domain, termed the Rev activation domain, is an ~10 amino acid leucine rich sequence that is critical for Rev function but dispensable for specific RNA binding. The activation domain has been shown to specifically bind a cellular, nucleoporin-like cofactor termed Rab or hRIP and also functions as an autonomous protein nuclear export signal (NES). Mutational analysis suggests that these three activities fully cosegregate, i.e. that the role of the Rev activation domain is to bind Rab which in turn induces the nuclear export of Rev and, hence, bound target RNA species. Functional domains equivalent to the Rev activation domain have recently been identified in TFIIIA, which is believed to function in the nuclear export of 5S rRNA and in PKI, which mediates the nuclear export of a cellular protein target. The PKI NES also binds Rab specifically and can, like the TFIIIA NES, fully substitute for the Rev activation domain in chimeric proteins. Therefore, it is clear that the Rev nuclear export pathway is used by multiple cellular factors and does not represent a dedicated RNA export pathway.

FOURTH SESSION

Chairperson: Angus I. Lamond

Interaction with influenza virus NS1 protein identifies hSTL, a human sequence homolog of *D. melanogaster* staufen protein

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The influenza virus NS1 protein is the only non-structural protein induced after infection. It is localized mainly in the nucleus, both in infected cells and when expressed from cloned cDNA, although a small fraction is present in the cytoplasm in association with polysomes [1]. Its expression leads to a series of effects on the viral and cellular transcripts: It mediates a generalized retention of polyadenylated RNA in the nucleus [2, 3] as well as a modulation in the splicing of pre-mRNAs [2, 4, 5]. In addition, it enhances the rate of translation initiation of viral, but not cellular, mRNAs [1, 6]. On the other hand, genetic evidence has suggested that NS1 protein is involved in the regulation of viral RNA transcription-replication [7]. These plethora of effects has been associated to the NS1 protein binding to U6 snRNA [5], polyA [3], virion RNA [8] and the 5' UTR of the viral mRNAs.

In an attempt to identify cellular factors that may play a role in the effects observed during NS1 protein expression, we have used the two-hybrid genetic trap to screen a human cDNA fusion library using NS1 protein as a bait. Partial sequencing of one of the interacting clones, isolated by its ability to induce the expression in yeast of the *his* and *lacZ* marker genes, showed a stricking homology to the *D. melanogaster* staufen protein. When used as a probe for northern analysis, the interacting clone detected a mRNA of 3-4 Kb in human cells. The ORF encoded in the interacting clone was expressed *E. coli* as a His-tagged protein and a specific antiserum was prepared. The expression of the interacting clone in human cells using the CMV promoter showed a cytosolic pattern of immunofluorescence staining reminiscent of a citoskeleton reticulum localization(as its Drosophila homologue). However, its co-expression with NS1 protein lead to a localization in nuclear foci that correlated strictly with the NS1 protein localization. In agreement with these results, the protein encoded in the interacting clone could be immunoprecipitated with anti-NS1 serum in extracts from cells co-expressing both proteins or in mixtures of extracts expressing either protein.

The coding sequence of the interacting clone was used as probe to identify the full-length cDNA from a λ phage library. Several λ clones were isolated and their sequence was determined. The presumable full-length clones encode a protein with 55% similarity to *D. melanogaster* staufen protein which we have named hSTL (human STaufen-Like). hSTL shows three of the domains responsible for dsRNA binding described in staufen and, within them, strictly conserve the residues described as essential for binding.

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mRNA Export From the Yeast Nucleus, A.M.Tartakoff,
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Proteins which interact with specific RNA sequences or conformational determinants have been implicated in mRNA export. Since most mRNAs do not obviously share structural determinants, except for the 5' cap (which appears not to be essential for export), these specific models may not be directly relevant to the export of the majority of mRNAs.

We postulate that there is a shared mechanism which causes export of most mRNAs and we therefore have sought to identify factors which are of broad importance for export. We also postulate that the mechanism of export can be divided into four steps: packaging into ribonucleoprotein particles, translocation within the nucleoplasm, specific recognition of nuclear pores, expulsion through nuclear pores. Components of the nuclear pore complex have previously been implicated in protein import and several mutations in pore proteins inhibit RNA export. It is not known to what extent these latter effects are direct.

To avoid certain of the complexities with are intrinsic to analysis of export, we have turned to model systems: the yeast *Saccharomyces cerevisiae* (in which the average polyadenylated RNA does not undergo splicing) and adenovirus (which causes its own mRNAs to be exported while inhibiting the export of host cell transcripts late during infection).

Using a genetic enrichment and screening procedures applied to yeast, we have obtained a family of 20 conditional temperature-sensitive mRNA transport (*mtr*) mutants which, at the restrictive temperature, accumulate poly (A) + RNA in the nucleus (1). All available mutants are recessive, several interrupt rRNA processing, but none show a ts splicing defect. Several of the corresponding genes have been cloned.

Mtr13p and Mtr4p may be important for the organization of the hypothetical mRNP. ***MTR13*** (= *NOP3, NPL3*) encodes a RNA-binding protein which resembles hnRNP A1 in that it shuttles in and out of the nucleus and includes both a pair of RNA recognition motifs and a carboxy-terminal glycine/arginine-rich domain. It shares a characteristic heptapeptide with the alternate splicing factors, ASF/SF2 and pSR55, and includes a putative nuclear export signal similar to that of hnRNP A1 (2). ***MTR4*** encodes a nuclear RNA-binding protein which includes a full set of "DEAD box" motifs as well as several leucine-rich putative nuclear export signals. This protein therefore may serve some normalizing function in the packaging or unpackaging of transcripts (3).

Mtr1p and Mtr7p govern nuclear pore function. ***MTR1*** (= *PRP20, SRM1*) encodes a homolog of a mammalian guaninine nucleotide

exchange factor, RCC1, a nuclear protein which regulates small GTPase(s) (Cnr1/2p = Gsp1/2p, homologous to Ran). This GTPase cycle is also regulated by the cytoplasmic protein, Rna1p. Ran itself interacts with nucleoporins in the context of protein import into the nucleus (4). ***MTR7*** (= ***ACC1***) encodes a cytoplasmic enzyme, acetyl CoA carboxylase, which is essential for synthesis of very long chain fatty acids. Upon loss of Mtr7p function, there are major alterations in the relation of nuclear pores to the outer membrane of the nuclear envelope (5).

Mtr2p and Mtr3p (as well as others) call into question the possible involvement of the nucleolus in mRNA export. ***MTR2*** encodes a novel nuclear protein which shows weak homology to proteins implicated in transfer of single stranded DNA between conjugating bacteria. Loss of function of Mtr2p (Mtr1p, Rna1p and others) causes the nucleolus to fragment and the accumulated poly (A) + RNA associates with these fragments (6). This fragmentation is seen only when RNA polymerase II is active, suggesting that the accumulated RNA itself causes fragmentation. ***MTR3*** encodes a novel nucleolar protein. Since *mtr3, mtr4, mtr14* and *mtr17* mutants cause poly (A) + RNA to accumulate in association with the nucleolus, we suggest that mRNPs have access to much of the nucleoplasm when their normal export is blocked and that the nucleolus finds such RNPs unexportable (7).

In our studies of adenovirus, we have shown that one of the two viral proteins which is responsible for the differential export phenotype which characterizes the late phase of viral infection, the E1B 55kDa protein, can inhibit poly (A) + RNA export when expressed in yeast (8). This observation suggests the existence of a corresponding evolutionarily shared target which is essential for export. It also argues that adenoviral transcripts are exported by some yet undefined path.

Ongoing studies indicate that some *MTR* gene products are also critical for protein export from the nucleus (9). We therefore suggest that these products define a general export path. Considering that some of these same genes are implicated in maturation of 20s-to-18s rRNA, they may be important for export of other classes of RNA.

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3'-end cleavage and polyadenylation of eukaryotic messenger RNA precursors

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The formation of the 3'-ends of nuclear pre-mRNAs leading to polyadenylated mRNAs proceeds in two main steps, endonucleolytic cleavage followed by polyadenylation. This simple reaction requires a surprisingly complex processing machinery. Purification and in vitro reconstitution of the cleavage and the polyadenylation reaction, as well as cDNA cloning and mutant analysis has allowed it to characterize many of the participating components and to establish their subunit composition. Cooperative interactions between the factors are important for processing complex assembly, RNA recognition, catalysis of poly(A) synthesis and the control of poly(A) tail length. Moreover, sequence comparisons of the mammalian 3'-end processing factors and their yeast homologs reveals basic similarities and important differences between lower eukaryotes and metazoans. A striking example of this are the poly(A) polymerases. These single-polypeptide enzymes have a modular sequence organization with different functional domains for primer binding, interaction with specificity factors, nuclear targeting and catalysis. Secondary structure prediction showed that their highly conserved catalytic core is similar to the palm domain of DNA polymerase b, the only protein of the family where the crystal structure is known. This homology extends as far as terminal deoxynucleotidyltransferase, cca: tRNA nucleotidyltransferase and streptomycin adenylyltransferase, an antibiotic resistance factor.

For reviews, see:
Keller, W.: No end yet to messenger RNA 3'processing! Cell 81, 822-832 (1995).
Wahle, E. 3'-end cleavage and polyadenylation of mRNA precursors. Biochim. Biophys. Acta 1261, 183-194 (1995).

mRNA TURNOVER IN YEAST

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To understand how mRNA decay rates are controlled, the mechanisms of mRNAs turnover and the gene products required for degradation need to be elucidated. Past experiments have identified three pathways of mRNA turnover in yeast. One pathway of mRNA degradation is initiated by shortening of the poly(A) tail, which triggers a decapping reaction, exposing the mRNA to 5->3′ degradation. Both stable and unstable transcripts degrade through this deadenylation-decapping pathway suggesting that it is common to many yeast mRNAs. There are also mechanisms of mRNA turnover that are related to, but distinct from, the deadenylation-decapping pathway. For example, the presence of an early nonsense codon in the PGK1 mRNA results in deadenylation-independent decapping. Similarly, blocking 5′ to 3′ degradation reveals a 3′->5′ pathway of degradation.

In order to understand how these pathways of mRNA degradation are controlled we have utilized several different genetic screens to identify gene products required for mRNA degradation. We have identified mutations in four genes, termed MRT1, MRT2, MRT3, and DCP1, that block or significantly slow the rate of mRNA decapping. By several different criteria the DCP1 gen encodes the decapping enzyme. Cells deleted for the DCP1 gene are viable, but grow slowly and show a block to mRNA decapping. In contrast, mrt1 and mrt2 mutations exhibit a temperature-sensitive lethal phenotype, suggesting these gene products may be essential. Interestingly the mrt1 and mrt3 mutations do not appear to affect deadenylation independent decapping. This observation suggests that these gene products may be primarily involved in modulating the rate of decapping on normal transcripts, perhaps by influencing mRNP structure. Current work is directed at understanding how these gene products interact to modulate the rates of mRNA degradation.

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"GENETIC ANALYSIS OF RNA-BINDING PROTEINS

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RNA-binding proteins (RNA-BPs) play a crucial role in a variety of cellular regulatory processes. Despite the biological importance of RNA-BPs and their key role in human health disorders, very little is yet understood about the structural features of these proteins that enable them to recognize their RNA targets with a high degree of discrimination. We have developed two genetic selection/screening procedures for rapidly identifying amino acid residues that are important for the binding affinity and specificity of RNA-BPs. One of these technologies is based on phage display and in vitro genetic selection; the other exploits *E. coli* genetics.

We have begun to use these methods to analyze RNA-BPs important for post-transcriptional regulatory processes in mammalian cells. Our initial studies have focused on the HIV-1 protein Rev and the mammalian spliceosomal protein U1A, each of which is a paradigm for a distinct family of structurally related RNA-BPs. These experiments have identified key roles for specific amino acid residues in target recognition. Our genetic data identifying likely contacts of specific Rev residues with particular RRE RNA nucleotides provide valuable clues as to how Rev docks in a sequence-specific manner with its RNA target. In the case of U1A, we have identified several amino acid residues important for RNA-binding specificity, one of which appears to play a novel structural role in locking the protein onto U1 snRNA. The genetic selection methods that we have developed promise to be useful both for structure-function studies of RNA-BPs and for the directed evolution of proteins specific for new RNA targets.

Laird-Offringa, I. A., and Belasco, J. G. (1995). *Proc. Natl. Acad. Sci USA* **92**: 11859-11863.

A nuclear cap-binding complex in pre-mRNA splicing and RNA nuclear export

E. Izaurralde, Joe D. Lewis, Chiara Gamberi and Iain W. Mattaj

We have characterized a conserved nuclear cap-binding protein complex (CBC). CBC is a heterodimer of two proteins, CBP80 and CBP20. Neither protein alone has affinity for cap structures, but the heterodimer binds specifically to capped RNAs. We have produced evidence that CBC is involved in two aspects of nuclear RNA metabolism, pre-mRNA splicing and RNA export from the nucleus.

In the case of splicing, in vitro data was obtained using extracts from which CBC had been immunodepleted. Removal of CBC from extracts severely reduced the efficiency of splicing of cap-proximal introns. Cap distal introns were much less affected, due to the presence of the polypyrimidine tract of the upstream intron, which apparently substitutes for the function of CBC.

The step of splicing that is affected by CBC depletion has been characterized using substrates with a single intron. In the absence of CBC, E (or commitment) complex formation is much reduced. A similar reduction in interaction between U1 snRNA and the 5' splice site can be seen in cross-linking. Thus, CBC appears to function to allow efficient recognition of the cap-proximal 5' splice site.

The second function of CBC is in RNA export from the nucleus. Of capped transcripts, those encoding the U snRNAs show a strong dependence on the cap structure for export. That this dependence reflects a requirement for CBC was shown directly by microinjection experiments. Antibodies that prevent CBC binding to capped RNAs, when microinjected into the nucleus, present U snRNA export. Although only indirect evidence supports the idea that CBC may also have a role in mRNA export, electron microscopic studies of Balbiani Ring RNPs in Chironomus tentans (in collaboration with N. Visa and B. Daneholt, Karolinska Institute) show that CBC associates with these pre-mRNAs early in their synthesis, consistent with the role of CBC in splicing, and also during passage of the RNPs through nuclear pore complexes to the cytoplasm, consistent with a role in RNA export. CBC staining is seen close to the nuclear pores, but only very weak staining is observed elsewhere in the cytoplasm, indicating that CBC is rapidly recycled to the nucleus after its export. The mechanism of recycling is under investigation and will be discussed.

FIFTH SESSION

Chairperson: Michael Rosbash

Translational Control by Cytoplasmic Poly(A) Elongation

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Early development in probably all metazoans is programmed by maternally-inherited mRNA. Generally, this material is translationally dormant in oocytes, but is activated in a sequence-specific manner during meiotic maturation, at fertilization, or during early embryogenesis. The translational recruitment of many of these mRNAs is regulated by cytoplasmic poly(A) elongation. In Xenopus and mouse oocyte maturation, two cis-acting sequences in the 3' UTRs of responding mRNAs are necessary for polyadenylation, the near-ubiquitous hexanucleotide AAUAAA, and the consensus octanucleotide UUUUUAAU. This second element is designated the cytoplasmic polyadenylation element, or CPE.

In Xenopus, the CPEs of all mRNAs tested that are polyadenylated during oocyte maturation are bound by a 62 kDa protein termed CPEB. These mRNAs include B4, G10, cdk2, c-mos, and cyclins A1, B1, and B2. The immunodepletion of CPEB from polyadenylation-proficient egg extracts renders all of these mRNAs incapable of being polyadenylated. Because c-mos RNA translation is known to be necessary for oocyte maturation, the importance of CPEB in this process was tested. Affinity-purified CPEB antibody injected into Xenopus oocytes not only abrogates polyadenylation of c-mos (and other) mRNA, but also prevents its translation. It is therefore not surprising that CPEB antibody also inhibits oocyte maturation. Neither control antibody nor IgG have any effect on these processes. One major conclusion from these studies is that CPEB is an essential component for development because it regulates the expression of c-mos mRNA.

Of the several mRNAs that undergo cytoplasmic polyadenylation during mouse oocyte maturation, again a particularly interesting one is that which encodes c-mos. In this case, however, c-mos mRNA polyadenylation and translation are necessary for progression from meiosis I to meiosis II. To explore the regulation of c-mos mRNA translation, a mouse CPEB cDNA has been cloned and sequenced. At the amino acid level, it is 80% identical to the Xenopus protein. Northern blotting and in situ hybridization show that mouse CPEB is present only in oocytes and testis tissue. Experiments are underway to assess whether this CPEB binds to the mouse c-mos CPE.

One extant question is how 3' poly(A) elongation promotes 5' translational initiation. In maturing Xenopus oocytes, an injected model RNA that is polyadenylated commensurately undergoes cap ribose methylation. This is a specific cap II methylation. When polyadenylation is prevented by mutating the important cis elements mentioned above, or by blocking chain elongation with cordycepin, cap ribose methylation does not occur. In addition, an in vitro 'preadenylated' RNA that cannot undergo further tail elongation because of a mutated cis element also is not cap ribose methylated. Therefore, the process of poly(A) elongation, and not a poly(A) tail per se, may be necessary for cap ribose methylation.

During Xenopus oocyte maturation and embryogenesis, some mRNAs are translated only when they are actively undergoing poly(A) elongation. This correlates with the data mentioned above (that cap ribose methylation occurs only when active polyadenylation takes place). When oocytes are incubated in the presence of SIBA, an analog of the methyl donor SAM that is a feedback inhibitor of methyltransferase reactions, poly(A) elongation is unaffected but cap ribose methylation is destroyed. Most importantly, there is no translational activation.

One model to explain the above results is that poly(A) polymerase, which interacts with the 3' end of a CPE-containing RNA during maturation, is complexed to a ribose methyltransferase, which in turn binds the 5' cap and catalyzes methylation. Because the oocyte cytoplasm contains mRNA that far exceeds its translational capacity, it is those mRNAs that offer a competitive advantage that are recruited onto polysomes. It may be that ribose methylated caps offer this advantage

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Translational regulation by mRNA binding proteins

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The binding of cytoplasmic proteins to specific sites in mRNAs mediates positive or negative translational control. During early animal development, particularly around fertilisation, the binding of regulatory proteins to the 3' UTR of several mRNAs induces lengthening of their poly-(A) tails and translational activation (1). Other RNA-binding proteins that respond primarily to metabolic or differentiation signals repress the translation of the specific mRNA(s) that they bind to. Examples of the latter group will be discussed.

Iron regulatory protein 1 (IRP-1) and IRP-2 bind to moderately stable hairpin structures (iron-responsive elements=IREs) in the 5' UTRs of ferritin, erythroid 5-aminolevulinate synthase, aconitase and succinate dehydrogenase mRNAs and repress their translation in iron-deficient cells (2). Repression appears to result from the steric inhibition of the association of the 43S translation preinitiation complex with the mRNA, and is negatively affected by increasing the distance between the IRE and the cap structure beyond ~60 nucleotides (3,4,5). Translational repression by this mechanism does not require a polyadenylated mRNA template in cell-free translation systems. However, mRNAs subjected to translational repression by this mechanism have a shortened poly-(A) tail in cultured cells. Poly-(A) tail shortening is not caused by the protein binding per se, but appears to reflect a consequence of the translational repression induced by the protein. While in the cases of positive translational control information flows from the 3' to the 5' end, these findings appear to reveal an example of information flow in the opposite direction (6).
The 15-lipoxygenase binding protein (LOX-BP) represents an example of a translational repressor protein that is regulated during erythroid cell differentiation and exerts its effect through a 3' UTR binding site. In cell-free translation extracts from rabbit reticulocytes, purified LOX-BP represses the translation of a non-polyadenylated 15-LOX mRNA (7), but little is know about the actual mechanism of translational repression. LOX-BP, purified by affinity chromatography, was microsequenced and cDNAs cloned. Recombinant proteins are currently being expressed in E. coli and functionally tested.

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mRNA 5' CAP FUNCTION AND REGULATION OF TRANSLATION.

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An important aspect of gene expression is the modulation of translation rates in response to growth factors, hormones and mitogens. Most of this control is at the level of translation initiation. All eukaryotic cellular mRNAs contain a cap structure (m^7GpppN) at their 5' terminus, which facilitates 40S ribosome binding to the mRNA. Cap function is mediated by the initiation factor eI4F, which consists of three subunits: a. eIF4E, the cap-binding polypeptide. b. eIF4A, an RNA helicase. c. eIF4G, formerly called p220, whose integrity is required for cap-dependent translation.

eIF4E plays a key role in regulating translation and in control of cell growth, and development. We have cloned cDNAs encoding two proteins, 4E-BP1 (eIF4E binding protein 1) and 4E-BP2, that bind specifically to eIF4E, and inhibit specifically cap-dependent translation initiation. Recently, we characterized two novel cDNAs that share homology with 4E-BPs. The interaction between 4E-BPs and eIF4E is dramatically diminished upon their phosphorylation in response to insulin and growth factors, concomitant with the relief of translational repression of capped mRNAs. Phosphorylation of 4E-BPs is effected by a rapamycin-sensitive signal transduction pathway, that is distinct from the MAP kinase pathway earlier implicated in the phosphorylation of 4E-BPs. PI3-kinase is an upstream component in this signal transduction pathway. Inhibitors of PI3-kinase, such as wortmannin, diminish the phosphorylation of 4E-BPs.

4E-BP1 competes with eIF4G for binding to eIF4E, through a binding motif that shares a common sequence among 4E-BPs and eIF4G. Thus, 4E-BPs prevent the formation of a functional eIF4F complex. We also demonstrated that phosphorylation of eIF4E by PKC *in vitro* occurs only after its dissociation from 4E-BP1. It is thus possible that phosphorylation of eIF4E *in vivo* is also dependent on its prior dissociation from 4E-BPs.

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IDENTIFICATION OF A REGION IN THE DOMAIN 3 OF THE APHTHOVIRUS IRES ESSENTIAL FOR INTERNAL INITIATION OF TRANSLATION.

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Translation of aphthovirus RNA is initiated at an internal ribosome entry site (IRES) element, preceding the first functional AUG initiation codon. A hypervirulent phenotype observed in a virus rescued after one hundred passages of persistent infection was associated to a 1.5 to 5-fold enhancement of the IRES activity relative to the parental virus IRES (1). The effect of mutations at the base of domain 3 of the aphthovirus IRES on translation activity has been analyzed by site directed mutagenesis, and expression of bicistronic RNAs in transfected cells. The results have shown that the enhanced IRES activity associated to a single pyrimidine transition fixed in the persistent aphthovirus variant is base-specific. Single or multiple mutations that did not affect predicted helical structures modified the relative efficiency of translation by at most 10-fold suggesting that primary sequence plays a role in IRES activity. However, mutations predicted to destabilize the base of domain 3 were detrimental to IRES function, showing up to 1000-fold reduction in the relative efficiency of internal initiation of translation. Subsequent restoration of the RNA structure in the defective IRES mutants gave rise to fully competent IRES, in some cases representing 7 nucleotide substitutions relative to the initial IRES element (2). A correlation between the energy of stabilization of the IRES structure and the efficiency of translation has been noted. None of the 15 mutations studied reached a level of initiation of translation comparable to that of the IRES from the persistent variant. Furthermore, mutations present in the persistent variant in the region around the first functional AUG initiation codon did not have a significant effect on the relative efficiency of internal initiation of translation. The results indicate a critical participation of the base of domain 3 in the activity of the aphthovirus IRES with a strong effect of secondary or higher order structures, and minor effects of primary structure.

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Picornavirus RNA translation: further evidence for *trans*- complementation of defective IRES elements.

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Picornavirus RNA is translated by a cap-independent mechanism. This process requires a region of about 450 nt from the 5' non-coding region which is predicted to fold into complex secondary structure. This element directs internal initiation of protein synthesis and is termed the Internal Ribosome Entry Site (IRES). The IRES activity is readily demonstrated by monitoring the translation of the second open reading frame within a bicistronic mRNA in which the IRES is the spacer between the two coding sequences. The IRES is normally considered a *cis*-acting element. However we have shown previously that mutant (and defective) IRES elements, within a bicistronic mRNA, can be complemented *in vivo* by the co-expression of an intact IRES element as a separate molecule. This process is efficient and requires a high degree of sequence identity between the two IRES elements, and hence presumably requires RNA-RNA interactions. The complementation process can be achieved by an IRES unlinked to any open reading frame but also when linked to a third reporter gene. Complementation has been demonstrated using IRES elements from three different genera of picornaviruses including the two major types of picornavirus IRES element. We have now shown that defective encephalomyocarditis virus (EMCV) IRES elements, with either deletions or point mutations within the three principal domains of this element, can be complemented by co-expression of just parts of the EMCV IRES. Thus defective IRES elements can be complemented by another defective IRES element. The 'helper' molecule must include at least the domain in which the defect occurs.

Identification, purification and cloning of the Tat coactivator, TA150.

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Expression of human immunodeficiency virus type 1 (HIV-1) genes is regulated by the *trans* activator Tat. Tat exerts its effects by increasing the rate of transcription, but the mechanism by which it does so is still unknown. To study the cellular factors required for Tat *trans* activation we have expressed functional Gst-Tat fusion protein and used it to construct affinity columns. Our findings are: i) a Gst-Tat affinity matrix depleted HeLa nuclear extracts of factor(s) required for Tat function. A Tat mutant bearing missense mutation (K41A) was incapable of this depletion; ii) Tat *trans* activation was recovered by adding unfractionated nuclear extract, the 0.5 M KCl elution fraction from the Tat affinity column, or sedimentation gradient fractions of HeLa extracts. The activity from the gradients sedimented with an apparent molecular mass of 140 to 200 kDa; iii) Tat trans activation could not be recovered using recombinant human TBP or partially purified TFIID, and iv) *trans* activation by Tat was blocked by heating the nuclear extract under conditions where basal transcription was not decreased. Our data demonstrate for the first time the existence of unique Tat co-activators distinct from factors required for general basal transcription.

The Tat co-activator was purified to near homogeneity using sequential K41ATat and Tat affinity columns. The co-activator migrated in SDS-PAGE gels with apparent molecular mass of 150 kDa and was shown to be a phosphoprotein. The 150 kDa Tat co-activator (TA150) was digested and several internal peptides sequenced. Using the peptide sequence information we have obtained cDNA clones that encode TA150. We have also obtained an anti-TA150 antiserum that detects the protein in Western assays and localizes the protein to the nucleoplasm of HeLa cells. We are using the cDNAs and the antiserum to confirm the role of TA150 as an essential Tat co-activator.

POSTERS

STUDIES ON THE POSTTRANSCRIPTIONAL REGULATION OF THE EXPRESSION OF THE MA16 PROTEIN FROM MAIZE

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The MA16 protein from maize is an abundant 16 KD RNA-binding protein that contains an RNA-binding domain (RBD or RMM) and a Gly-Arg-rich domain[1]. Its main localization is the nucleolus, with a predominance in the dense fibrillar component (DFC), as observed by immunoelectron microscopy[2]. We have also shown,by cell fractionation studies, that it is a non-ribosomal protein[2]. Potential candidates to interact with this protein are the rRNA and snoRNAs. Immunoprecipitation studies have revealed a number of small RNAs that are coprecipitated with an anti-MA16 polyclonal serum.

The expression of MA16 in different maize tissues has been analyzed by *in situ* hybridization. These studies have shown that it is specially abundant in elongating and expanding structures, such as the root elongation zone and young leaves. These data, together with its nucleolar localization, seem to indicate that this protein is involved in general growth phenomena in the plant.

We have also studied the expression of MA16 under a number of environmental stresses, such as hydric stress or the adhition of exogenous abscisic acid (ABA). The increase of the mRNA observed under such conditions does not seem to be followed by an increase in the protein abundance. ABA is a growth inhibitory vegetal hormone, and it has been reported that its exogenous addition causes a general repression of protein synthesis. These observations have raised the possibility of a post-transcriptional repression of the expression of MA16 in growth-arrested conditions. The analysis of the MA16 mRNA leader has revealed a strong secondary structure, acording to primer extension data and computer prediction. By in vitro studies we have observed that the presence of the whole leader sequence causes an inhibition of the translation of the adjacent open reading frame, when compared with transcripts bearing only a small fragment of this leader together with polylinker sequences. Moreover, a stretch of pyrimidines (12-17 nucleotides long), that has been found in this leader, has also been observed in all the leader sequences of the mRNAs of the 10 plant MA16-homologue proteins cloned up to date. The conservation of this pyrimidine-stretch seems to indicate that it has a role in the regulation of the expression of these proteins. Interestingly, a stretch of pyrimidines present in the 5'end of a number of mammalian ribosomal protein mRNAs has been involved in the repression of their translation under growth-arrested conditions.

To study whether the leader sequence of the MA16 mRNA is responsible for a translational repression of this protein in growth-arrested conditions, we are going to fuse different fragments of the leader with a reporter gene (GUS protein), and transiently transform different ABA-treated and non-treated maize tissues. We would also like to compare the results we get with constructs made using the leader sequence of the rab28 protein from maize (selectively expressed with ABA), in an attempt to find out the reasons for their different translability in various growth states.

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Nonsense mutations inhibit splicing of immunoglobulin k-light chain mRNAin a cell free system: Recognition of mutant codon is independent of protein synthesis and is tissue specific.

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The expression of antibody genes involves DNA rearrangements often leading to frame shifts, the affinity maturation of the immune response depends on a processes of hyper mutation which can generate premature stop codons. Premature termination of protein synthesis leads to defective protein products which can cause disorders due to the loss of function or by a dominant negative mechanism. To overcome such possibility nonsense mutations in this and in other systems also result in the down regulation of the mRNA level (Maquat, 1995; Lozano et al. 1993). Lozano et al. (1994), using an in vivo approach have shown that the pool of mRNA is not depleted by an increase in cytoplasmic or nuclear mRNA decay. On the contrary, an apparent increase in unspliced form suggests that an alteration of splicing is involved in the mRNA down regulation.

We have now developed a cell free assay, using nuclear extracts, which shows that nonsense mutations in antibody light chains result in defective splicing and not RNA decay. This cell free system correlates with the in vivo down regulation of k-light chain mRNA harbouring nonsense mutations (Aoufouchi et al, 1996) and therefore appears to be a powerful tool to analyse the molecular basis of such mechanism overcoming the limitation and complications of in vivo experimental systems used so far. We have shown also that splicing deficiency does not depend on the sequence environment of the nonsense codon or with the codon itself, but can be partially corrected by mutating the methionine initiation codon. Despite the apparent link between translation and low mutant mRNA levels, inefficient splicing is not dependent on protein synthesis. The splicing deficiency is B-cell specific, since it is observed with both human and mouse B-cell extracts but not with HeLa or T-cell extract.

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Expression of the protein kinase PKR is modulated by IRF-1 and is reduced in 5q-associated leukemias.

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The transcription factor IRF-1 which has been shown to be deleted in a number of human leukemias exhibits tumor suppressor activity. Its overexpression results in inhibition of cell growth. The double-stranded RNA-dependent protein kinase (PKR), contains a promoter element for the binding of IRF-1 and exhibits antiproliferative properties. We investigated the role of IRF-1 in PKR expression. In IRF-1-deficient embryonic fibroblasts, PKR expression was found to be reduced 4 fold relative to wild-type cells. This finding suggested that diminished expression of PKR could result from deletion of the IRF-1 gene in human leukemias. We analysed the human leukemic U937 cell line which we demonstrated to contain a deletion of one IRF-1 gene. U937 cells were found to contain reduced levels of PKR relative to other leukemic cell lines with two copies of IRF-1 gene. Transfection of IRF-1 into U937 cells, strongly induced PKR expression. PKR levels were determined in leukemic cells from patients with transformed myelodysplasias. PKR was markedly reduced in cells carrying a deletion of chromosome 5q, a locus to which IRF-1 was mapped. Therefore, PKR is a strong candidate for a mediator of the tumor suppressor activity of IRF-1.

Rapamycin blocks the phosphorylation of 4E-BP1 and inhibit cap-dependent initiation of translation.

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The immunosuppressant drug rapamycin blocks progression of the cell cycle at the G1 phase in mammalian cells and yeast. We showed that rapamycin inhibits cap-dependent, but not cap-independent translation, in NIH 3T3 cells. Cap-dependent translation is also specifically reduced in extracts from rapamycin-treated cells as determined by *in vitro* translation experiments. This inhibition is causally related to the dephosphorylation and consequent activation of 4E-BP1, a protein recently identified as a repressor of the cap-binding protein, eIF-4E, function. These effects of rapamycin are specific, as FK506, a structural analogue of rapamycin, had no effect on either cap-dependent translation or 4E-BP1 phosphorylation. The rapamycin-FK506-binding protein complex is the effector of the inhibition of 4E-BP1 phosphorylation as excess of FK506 over rapamycin reversed the rapamycin-mediated inhibition of 4E-BP1 phosphorylation. Thus, inactivation of eIF-4E is, at least partly, responsible for inhibition of cap-dependent translation in rapamycin-treated cells. Furthermore, these results suggest that 4E-BP1 phosphorylation is mediated by the FRAP/TOR signalling pathway.

NUCLEOLIN IS A SEQUENCE-SPECIFIC RNA BINDING PROTEIN: CHARACTERIZATION OF TARGETS ON PRE-RIBOSOMAL RNA

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Nucleolin is an abundant nucleolar protein which plays an essential, but largely unknown role in ribosome biogenesis. Nucleolin contains four consensus RNA binding domains (CS-RBD), presence of which suggests that the molecular function of this protein is likely reflected by its RNA binding properties. Indeed, by immunocytological analysis performed on ribosomal transcription units and RNA-protein cross-linking assays, we have found several nucleolin molecules being associated with nascent pre-rRNA. The binding sites on the pre-rRNA have been predominantly localized to the 5' end of the 5' external transcribed spacer (5'ETS), to the coding regions of 18S and 28S rRNAs and to the 3' external transcribed spacer (3'ETS). In mouse, two high affinity binding sites with an apparent dissociation constant (K_d) of 50-100 nM have been mapped in the 5'ETS upstream to the early pre-rRNA processing site. Interestingly, nucleolin of mouse origin has recognized analogous sequences in the 5'ETS of rat and human pre-rRNA. In parallel, *in vitro* selection-amplification (SELEX) experiments have identified an 18 nucleotides-long RNA sequence that binds nucleolin with high affinity (K_d 5-20 nM) and shares a common UCCCGA motif with the characterized pre-rRNA binding sites.

However not all U/GCCCGA-containing RNAs were recognized by nucleolin. By mutagenesis and a structural analysis, we have characterized the nucleolin cognate RNA binding site and found that it is constituted of a minimal 18-nucleotides long RNA hairpin structure. The sequence U/GCCCGA found within the hairpin loop is necessary for the specific interaction. Mutation of any of the C or G residues within this motif abolish nucleolin interaction. Furthermore, point mutation in the stem that disrupt completely the hairpin structure also prevent nucleolin binding. By determining the minimal 5' and 3' end of the RNA that is bound to the protein we concluded that nucleolin binding site is constituted by a 18-nucleotide long RNA composed of a short 4-5 base paired stem and an 8 nucleotides loop. To conclusively demonstrate that this small RNA was sufficient for nucleolin interaction, we used a chemically synthetized RNA corresponding to a stem-loop structure of a natural binding site. Indeed, this small RNA interacted specifically with nucleolin. This structural motif is very similar to hairpins recognized by two other CS-RBD containing proteins (U1-snRNP A and U2-snRNP B") and is likely to be shared by other CS-RBD cognate RNA.

CHARACTERIZATION OF POLYADENYLATION-COMPETENT NUCLEAR EXTRACTS FROM MOUSE B-CELL LINES, **G. Edwalds-Gilbert and C. Milcarek, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261**

During the development of mouse B cells there is a regulated shift from the production of membrane to secretory-specific forms of immunoglobulin mRNA, which predominate in the late stage/plasma B cells. By DNA transfection experiments we have shown that *in vivo* there is an increase in the efficiency of the cleavage-polyadenylation reaction which accompanies the developmentally regulated shift to secretory-specific forms of immunoglobulin mRNA. When we look *in vitro* at polyadenylation competent nuclear extracts prepared from early versus late stage/plasma B cells, we see cell stage-specific changes in the binding activities of two general polyadenylation factors, the 64 kDa subunit of CstF and the 100 kDa subunit of CPSF (Edwalds-Gilbert and Milcarek, Mol. Cell. Biol. 15:6420-6429 and Nucl. Acids Symp. 33:229-233). The increased binding is seen on both immunoglobulin secretory and SV40 substrates, implying that increased use of the immunoglobulin secretory poly(A) site in plasma cells is due to a generalized change in polyadenylation in the plasma cells. We are attempting to identify factors from B cell and plasma cell extracts that confer differential binding and activity of the 64 kDa and 100 kDa proteins to poly(A) sites. Characteristics of the B cell and plasma cell nuclear extracts will be presented.

Stability and transcriptional termination of *Kluyveromyces lactis KlCYC1* mRNAs

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The yeast *Kluyveromyces lactis KlCYC1* gene encodes for a protein that presents a high homology with iso-1-cytochrome *c* protein in *S. cerevisiae*. Despite to this fact, at the transcriptional level, there are two significant differences between *CYC1* and *KlCYC1* genes: **i)** *KlCYC1* produces two transcripts that are bigger in size than the unique *CYC1* mRNA and **ii)** *KlCYC1* transcripts are present at higher level than *CYC1* mRNA. This last feature also correlates with the physiological diferences between this two species, since *S. cerevisiae* is a predominantly fermentative yeast, even in full aerobic conditions, and *Kluyveromyces lactis* uses respiration more eficiently under the same conditions (1,2).

There is some differential stability between these two messengers, in this sense the 1.2 transcript seems to be more stable than the 1.6 one. Northern experiments using poly(A) RNA confirmed that both *KlCYC1* transcripts correspond to polyadenilated forms. Interestingly S1-mapping analysis revealed the existence of only one principal transcription start site and two major end-points placed at positions 1370 and 1070 (respect to the Hind III site of the ORF) which explain the origin of the two transcripts (2). This is also an important difference with *CYC1* since this gene contains a major termination signal.

Several sequences have been identified in yeast that function to specify transcription termination but a general consensus which could be extended to all yeast genes has not been found yet. The tripartite sequence TAG...TA(T)GT...TTT is essential for 3´end formation of *CYC1* mRNA and for another *S. cerevisiae* genes as *ADH2, ARO4*, *TPR1* and *TRP4*. An AT repeat is also implicated in the termination of the *ScCYC1* transcription (3).

Analysis of *KlCYC1* 3´sequence showed the presence of multiple consensus with these termination signals. In heterologous expression experiments we have shown that when *S. cerevisiae* is transformed with *KlCYC1* gene the presence and the size of the two transcrips is mantained in this yeast

To ascertain the possible relevance of maintainig two transcripts with a big 3´end we are performing northern experiments comparing the differential stability under different stages of culture growth and under conditions blocking *KlCYC1* transcription.

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Translational control of mouse c-*mos* mRNA

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The c-*mos* proto-oncogene encodes a serine-threonine kinase that is expressed almost exclusively in vertebrate germ cells, where it participates in the regulation of meiosis. In oocytes, c-*mos* is transcribed as a maternal mRNA that is stored during oogenesis and is translated upon initiation of meiosis. We have shown that this translation event is regulated by cytoplasmic polyadenylation in mammals (1). Cytoplasmic polyadenylation of mouse c-*mos* mRNA requires three cis elements in the 3' UTR: the hexanucleotide AAUAAA and two U-rich cytoplasmic polyadenylation elements (CPEs). In order to understand the mechanism of translational control by cytoplasmic polyadenylation, we have attempted to isolate the factor(s) that bind to the CPEs of mouse c-*mos* mRNA. As a first approach, we have obtained the mouse homolog of CPEB, a protein that recognizes the CPEs of several mRNAs in Xenopus and is required for their polyadenylation. The mouse protein, mCPEB, is 80% identical to its Xenopus counterpart. The highest homology is found in the carboxy-terminal half, which contains the putative RNA binding domain. mCPEB mRNA is present primarily in ovary, testis and kidney. Within the ovary, the mRNA is exclusively located to the oocytes. Experiments addressing the binding of mCPEB to the 3' UTR of c-*mos* mRNA and its role in polyadenylation are in progress.

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Structure of the Complex Between the Human U1A Protein and a Conserved RNA Element in the U1A Pre-mRNA

The U1A protein binds to its own pre-mRNA in the 3' untranslated region (UTR), and prevents polyadenylation of the pre-mRNA by interacting with poly(A) polymerase. The binding sites for U1A in the 3' UTR of its own pre-mRNA are a pair of internal loops in an extended helical structure. One protein molecule binds to each loop. We have determined the structure of one of these binding sites alone, the structure of the N-terminal RNP domain of U1A, and the complete RNA-protein complex between them. These three structures provide the basis for an unusually detailed understanding of the principles of RNA-protein recognition by RNP domains. Examining the RNA structures, seven RNA nucleotides which interact with the protein are in the same conformation in the free and bound RNA, and the other five are flexible until the complex is formed. The free and bound protein structures show that two variable loops are in the same conformation in the free and bound protein. The rest of the RNA-binding surface is covered by an α helix which must move out the the way for the RNA to bind. The parts of the RNA and the protein which do not change conformation during binding interact with one another in the complex. This implies that complex formation involves two main steps: 1) the rigid body interaction between the RNA and the protein involving the invariant elements of structure, and 2) the reorientation of the α helix and the folding of the flexible unpaired nucleotides onto the newly exposed RNA binding surface. We have used this structure to model the interaction between two U1A proteins and the full length 3' UTR. This model suggests that protein protein interactions involving the C-terminus of each monomer may occur in the trimolecular complex, and that two U1A proteins together form a binding site for poly(A) polymerase.

CHARACTERIZATION OF NOVEL INITIATION FACTOR (eIF)-2α KINASES FROM *Drosophila* EMBRYONIC AND MOUSE LIVER CELLS

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Protein synthesis is regulated in response to environmental stimuli by covalent modification, primarily phosphorylation, of components of the translational machinery. Phosphorylation of the α subunit of eIF-2 is one of the best-characterized mechanisms for down-regulating protein synthesis in mammalian cells in response to various stress conditions. In *Drosophila* such regulatory mechanism has not been elucidated.

Three distinct protein kinases regulate protein synthesis in eukaryotic cells by phosphorylating the α subunit of eIF-2 at serine-51. They are human PKR, rabbit HCI and yeast GCN2. The regulatory mechanisms and the molecular sizes of these eIF-2α kinases are very different.

PKR is induced by interferon and activated by low concentrations of dsRNA. HCI is activated under heme-deficient conditions and its kinase activity is inhibited by heme. Yeast GCN2 is activated by amino acid starvation.

The phosphorylation of eIF-2α results in the shut-off of protein synthesis. Nevertheless, the eIF-2α kinases can regulate both global as well as specific mRNA translation.

We have reported the identification and characterization of p69, a *Drosophila* eIF-2α kinase which phosphorylates the α subunit of rabbit reticulocyte eIF-2. This study constitutes the first identification of an eIF-2α kinase in a high eukaryote distinct from mammals.

Anti-HCI-peptide antibodies recognizing HCI, but not PKR, immunoprecipitate an eIF-2α kinase activity and recognize a 69 kDa polypeptide in Western blots of *Drosophila* Schneider's cell line S3 extracts. *Drosophila* eIF-2α kinase interacts with hsp83, a *Drosophila* heat shock protein related to the mammalian hsp90 that interacts with HCI. *Drosophila* eIF-2α kinase changes its subcellular localization in response to heat-shock. In addition, *Drosophila* eIF-2α kinase is selectively expressed in a group of midline cells in the embryonic nervous system, suggesting a role for this kinase in differentiation. Because *Drosophila* eIF-2α kinase appears to be heme-insensitive, p69

could be a new eIF-2α kinase which become activated in response to heat shock and other stress conditions.

We have developed an improved cell-free protein synthesis system derived from mouse liver which faithfully translates endogenous mRNA. The system is capable of re-initiation as demonstrated by inhibition of translation with edeine. We have also reported the identification and characterization of a novel mouse liver eIF-2α kinase (MLK) related to the HCI family of mammalian eIF-2α kinases. Like HCI, mouse eIF-2α kinase is heme-sensitive and interacts with mammalian hsp90.

Interestingly, mouse liver post-mitochondrial supernatant contain an inhibitor of both HCI and mouse eIF-2α kinase (MKIF) that could be involved in translational control in mammalian liver. The main characteristics of this novel inhibitor are the following : a) Inhibits HRI, PKR and MLK activities; b) Is sensitive to N-ethylmaleimide and to trypsin; c) Does not inhibit PKA, PKC or CKII activities; and d) Does no contain an eIF-2αP phosphatase activity.

ASPETCS OF THE TRANSLATIONAL CONTROL OF 5' TERMINAL OLIGOPYRIMIDINE mRNAs.

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The group of 5' terminal oligopyrimidine (TOP-) mRNAs includes messengers coding for vertebrate ribosomal proteins and other components of translation apparatus, like elongation factor (EF) 1α and EF2. The translation of TOP-mRNAs is regulated, in general, according to the growth status of the cell by rapidly changing the fraction of mRNA associated with polysomes. Localization changes of TOP-mRNA have been shown in many eukaryotic cultured cell systems following growth rate variations, as well as in amphibian and mammalian in vivo systems during development. Sequence elements involved in translational control have been identified in the 5' UTR of TOP-mRNAs. In particular the polypyrimidine stretch at the 5' end of the messengers is necessary although probably not sufficient.

During the analysis of TOP-mRNA translational control in Xenopus cultured cell, we observed that treatment of cells with transcription inhibitor actinomycin D interfered with the mRNA localization change. To further analyze the phenomenon we have used different drugs and dosages to inhibit transcription both totally and partially in the following ways: a) total transcription inhibition with high doses (2μg/ml) of actinomycin D or cordycepin; b) polymerase I transcription inhibition with low doses (50 ng/ml) of actinomycin D; c) polymerase II transcription inhibition with α-amanitin or dichloro-beta-D-ribofuranosylbenzimidazole (DRB). The results show that total transcription inhibition can relieve translational repression of TOP-mRNAs in cultured cells. In fact treatment of serum-starved cells with high doses of actinomycin D or cordycepin induces a localization change of TOP-mRNAs from mRNPs to polysomes. Since the effect is not observed in the presence of pol I or pol II inhibitors we conclude that the phenomenon is due to pol III inhibition. One possibility is that translation repression of TOP-mRNA requires, directly or indirectly, a pol III product and that transcription inhibition causes a decrease in the amount of such repressor.

More recently it has been shown that rpS6 phosphorylation by p70 S6 kinase in response to mitogenic stimulation parallels translational activation of 5' TOP-mRNA. To establish the possible causal relationship between these two phenomena, in collaboration with Geroge Thomas at the Friedrich Miescher Institute in Basel, I am using *in vitro* translation extracts prepared from HeLa cells. Extracts are prepared from both growing (5' TOP-mRNAs translationally active) and resting cells (5' TOP-mRNAs translationally repressed). The role of rpS6 will be investigated by altering its phosphorylation status in the extracts and monitoring the consequences on 5' TOP-mRNA translation. In particular a constitutively active form of p70S6k will be tested for its capacity to stimulate 5' TOP-mRNA translation in the extract from resting cells.

MOLECULAR ANALYSIS OF INFLUENZA VIRUS NS1 PROTEIN

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The genetic material of influenza virus consists of eight negative-stranded RNA segments. RNA segment 8 encodes two proteins, NS1 and NS2, by differential splicing. The collinear transcript acts as a mRNA for NS1 protein, while the spliced mRNA encodes NS2 protein. The non-structural protein NS1 accumulates mainly in the nucleus during virus infection. It is a RNA-binding protein that can bind both vRNA and poly A^+-RNA. It has been described that NS1 expression leads to an inhibition of mRNAs nuclecytoplasmic transport, and therefore an accumulation of mRNAs in the nucleus. Furthermore, inactivation of the NS1 gene increases the splicing efficiency of its own pre-mRNA, suggesting that NS1 is a inhibitor factor of pre-mRNA splicing. This effect is not specific since NS1 protein also inhibits the splicing process of non-viral pre-mRNAs.

To identify functional domains of this protein, we have constructed three types of mutants: amino-terminal deletion mutants, carboxy-terminal deletion mutants and amino acid insertion mutants. *In vivo* expression and labeling of the mutants showed that all of them are expressed in amounts similar to wt NS1.

To assay the ability of the mutants to retain mRNAs in the nucleus, two different approaches have been carried out. First, cultures of COS-1 cells were transfected with vectors co-expressing the mutated proteins and influenza virus NP protein. After fractionation into nuclear and cytoplasmic fractions, polyA$^+$ RNAs were isolated and analyzed by dot-hybridization using a NP-specific probe. Second, cultures of HeLa cells were transfected with plasmids expressing the mutated proteins. After fixation and incubation with a fluorescent-labeled anti-polyA$^+$ oligonucleotide, polyA$^+$ mRNAs localization was shown by fluorescence microscopy. Preliminar results of both assays suggest that aminoacids 1 to 113 are sufficient to inhibit the mRNAs nucleocytoplasmic transport.

REGULATION OF uPA GENE EXPRESSION DURING MYOGENESIS: EVIDENCE FOR POST-TRANSCRIPTIONAL REGULATORY MECHANISMS.

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The urokinase-type plasminogen activator (uPA) is a serine protease involved in several physiological and pathological events such as fibrinolysis, embryo morphogenesis, cell migration, invasiveness and tissue remodelling. The aim of our work has been to elucidate whether uPA is involved in the process of myogenesis.
C2C12 cells are murine skeletal myoblasts which can be manipulated to fuse and differentiate by changing the culture conditions. There was a correlation between the levels of uPA activity and the extent of myotube formation, with uPA mRNA increasing dramatically prior to myoblast fusion. To asses whether the uPA mRNA increase was due to transcriptional regulation of the gene, we performed run-on assays at the proliferating, confluent and differentiated states, as well as analysis of promoter deletion contructs linked to the luciferase reporter gene in stably transfected cells. The run-on transcriptional rates were similar throughout the whole myogenic process, therefore suggesting that post-transcriptional mechanisms might account for the uPA mRNA increase. Nevertheless, luciferase constructs (-8,2 Kb and -2 Kb) were only active in proliferating cells, thus implying the relevance of other gene sequences in the transcription of uPA in differentiated cells. Stability of transcripts was studied by DRB chase experiments in proliferating and differentiated cells. The uPA mRNA half-life was found to be different at both states, being more stable in the proliferating cells. uPA mRNA 3'untranslated region contains an AU-rich region close to the polyadenylation signal. In other mRNAs, AU-rich regions have been reported to bind to cytosolic proteins which take part in the modulation of mRNA turn-over. We tested the possibility that one of such AU-rich binding proteins (AUBP) might be present and differently expressed in the proliferating and differentiated muscle cells, by RNA band-shift and UV cross-linking assays. A protein of 48 kd was shown to bind uPA mRNA AU-rich region. However, this protein was detected in both the proliferating and differentiated muscle cells. We are currently investigating the role of this protein in post-transcripcional regulatory mecanisms other than mRNA stabilization, such as the control of transcripcion and translation.DGICYT PM92-178

The elongation factor Ts from *T. Thermophilus*

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The gene of elongation factor Ts from *Thermus thermophilus* was sequenced, cloned and the protein overproduced in *E. coli*. In comparison with EF-Ts from *E. coli* with 282 amino acid residues, EF-Ts from *T. thermophilus* is considerably shorter, differing by 86 amino acids. EF-Ts from the thermophile is stable at high temperatures, which facilitates its separation from *E. coli* proteins. Purified *T. thermophilus* EF-Ts forms a homodimer which contains a disulfide bridge between the two cysteines at position 190. Modification of cysteine 190 by iodoacetamide affects neither dimerization nor the ability of EF-Ts to facilitate the nucleotide exchange of EF-Tu. The disulfide bridge was detected only in purified EF-Ts, but not in protein extracts immediately after cell disruption. The physiological role of this S-S bridge remains therefore unclear. Some EF-Ts variants were obtained that are no more able to form dimers. These monomers seem to be inactive in acceleration of guanine nucleotide exchange in EF-Tu. Besides the tetrameric $EF\text{-}Tu_2 \cdot EF\text{-}Ts_2$ complex, a trimeric $EF\text{-}Tu \cdot EF\text{-}Ts_2$ complex can be detected by gel permeation chromatography and polyacrylamide gel electrophoresis. Trypsin cleavage after lysine 48 or modification of Cys78 yield inactive EF-Ts that does not bind to EF-Tu, but is still capable of forming homodimers.

SEQUENCE AND STRUCTURAL ELEMENTS IN THE POLYADENYLATION SIGNAL OF YEAST *FBP1* GENE

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Inverted repeats potentially able to form cruciforms have been found in many different organisms, specially in promoters and regions of transcription termination in bacteria and viruses. Although the existence of cruciforms in superhelical DNA *in vitro* has been demonstrated in many cases, the presence of these structures *in vivo* in eukaryotes is less certain. We have showed that an imperfect $(dA\text{-}dT)_{12}$ tract placed at the 3' end of the *FBP1* gene exists as a cruciform in naked DNA depending on the supercoiling state (1). Using nested deletions, *in vitro* processing, and an *in vivo* functional assay (2) we have found that a short sequence including the $(dA\text{-}dT)_{12}$ tract acts as a very efficient polyadenylation signal and strong terminator in both directions. This sequence overlaps also with sequences proposed for mRNA 3' end formation in yeast (3, 4) and for efficient transcription termination (5, 6). The complete deletion of the sequence capable of cruciform formation leads to the detection of very low levels of transcript although we still detect processing of this transcript with different assays (2, 7). Determination of the polyadenylation sites in this case by RACE indicates that the specificity has completely disappeared and many sites are used randomly. These results rise the possibility of a functional implication of the A+T rich sequence by itself or the non-B-DNA structure, or mRNA secondary structure derived from it in the 3' end formation in yeast.

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Alternative splicing of exon 14 determines nuclear or cytoplasmic localisation of FMR1 protein isoforms

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Impaired expression of the FMR1 gene is responsible for the fragile X mental retardation syndrome. The FMR1 gene encodes a cytoplasmic protein with RNA-binding properties. Its complex alternative splicing leads to several isoforms, whose abundance and specific functions in the cell are not known. We have cloned in expression vectors, cDNAs corresponding to several isoforms. Western blot comparison of the pattern of endogenous FMR1 proteins with these transfected isoforms allowed the tentative identification of the major endogenous isoform as ISO 7 and of a minor band as an isoform lacking exon 14 sequences (ISO 6 or ISO 12), while some other isoforms (ISO 4, ISO 5) were not expressed at detectable levels. Surprisingly, in immunofluorescence studies, the transfected splice variants that exclude exon 14 sequences (and have alternate C-terminal regions) were shown to be nuclear. Such differential localisation was however not seen in subcellular fractionation studies. Analysis of various deletion mutants suggests the presence of a cytoplasmic retention domain encoded in exon 14, and of a nuclear association domain encoded within the first 8 exons that appear however to lack a typical nuclear localisation signal.

THE *ARO4 /HIS7* INTERGENIC REGION: HOW DOES THE YEAST CELL AVOID TRANSCRIPTIONAL INTERFERENCE.

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The *ARO4* gene of *Saccharomyces cerevisiae* codes for the tyrosine inhibited 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase catalyzing the first step in the shikimate pathway (Künzler *et al.* 1995). The *HIS7* gene is located just downstream of the *ARO4* gene on the yeast chromosome II. It codes for the bifunctional glutamine amidotransferase:cyclase catalyzing the fifth and sixth step in the *de novo* histidine biosynthesis (Künzler *et al.* 1993). The two genes are transcribed in the same orientation. The intergenic region between the UAG stop codon of the *ARO4* gene and the AUG start codon of the *HIS7* gene consists only of 418 bp. Within this short interval the yeast cell has to terminate *ARO4* transcription and to initiate transcription of the *HIS7* gene.

We have analyzed this intergenic region in detail. The *ARO4* 3'end formation signal belongs to the class of weak bidirektional terminators (Irniger *et al.* 1991). It is at least 70 pb in length and contains a Zaret/Sherman sequence element. We are currently characterizing this poly(A) addition site in more detail using our 3'end formation test system containing an *ACT* promoter and an *ADH1* terminator with a multiple cloning site inbetween (Irniger *et al.* 1991). This test system allows us to address the question how transcriptional interference between the two genes is avoided. There are now some evidences that a 16 bp element directs 3'end formation on the *ARO4* transcript in both orientations. It is located just 10 bp upstream of the most proximal poly(A) site and does not contain the Zaret/Sherman sequence. In addition, deletion of the Zaret/Sherman element has no effect on *ARO4* 3'end formation in both orientations. Downstream of the 16 bp sequence we found some cis-acting elements which do not affect *ARO4* 3'end formation but regulate *HIS7* transcription. However Deletion of the 16 bp sequence has no effect on *HIS7* transcription. This result suggests that the cell efficiently prevents transcriptional interference between the *ARO4* and the *HIS7* gene even in the case of a non functional *ARO4* polyadenylation site. We are now addressing the question how the cell avoids transcriptional interference.

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SEQUENCE-SPECIFIC CONTROL OF PRE-mRNA METABOLISM IN YEAST BY THE PRODUCTS OF THE *NRD1* AND *NRD2* GENES

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We have identified a *cis*-acting element and two *trans*-acting factors that modulate gene expression in the yeast *S. cerevisiae* by altering pre-mRNA accumulation. The *cis*-acting element is an artificial construct about 140 nucleotides long and consists in part of anti-sense U6 RNA. Introduction of the sequence element into the intron of a reporter gene results in a 20-fold decrease in the steady-state amount of reporter pre-mRNA. The decreased abundance of full-length pre-mRNA is accompanied by the appearance of 3'-truncated pre-mRNA fragments whose 3' ends lie downstream of the element insertion site. Point mutations in a 14-nucleotide region of the anti-sense U6 RNA greatly diminish the element's effect, and may define a recognition site for a binding factor. Deletion studies indicate that features flanking this sequence-specific region are also required for the down-regulation of pre-mRNA abundance. The generation of truncated pre-mRNA fragments is consistent with either premature transcriptional arrest, or RNA degradation via endo- and/or exonucleolytic digestion.

Extragenic mutations that suppress the effect of *cis* element insertion arise spontaneously in two genes, which we have designated *NRD1* and *NRD2* (for Nuclear pre-mRNA Down-regulation). We have cloned the *NRD1* gene and found it to encode a novel yeast hnRNP-like protein with several interesting features. These include a putative RNA binding domain of the RNP consensus type, a region rich in RE and RS dipeptides similar to those found in a number of proteins involved in constitutive and regulated splicing in metazoans, and a C-terminal proline + glutamine rich (P+Q) domain similar to that of the yeast hnRNP protein, Nab3 (1). We hypothesize that the Nrd1 protein binds directly to the *cis* element in pre-mRNA (perhaps to the 14-nucleotide region encompassing the suppressor point mutations) and that this binding is a prerequisite for the generation of truncated pre-mRNA fragments. The RE/RS domain of Nrd1 suggests that this pre-mRNA control mechanism may be coupled to the splicing pathway.

We have also cloned the *NRD2* gene, which appears to be identical to *SEN1*. Sen1 is an essential nuclear protein that has a domain sharing homology with the helicase motif of the yeast Upf1 protein, which is involved in cytoplasmic nonsense-mediated mRNA decay (2-4). Mutations affecting *SEN1* have pleiotropic effects on a variety of nuclear functions, including tRNA splicing, rRNA processing, localization of nucleolar proteins, chromosome maintenance, and nuclear fusion (2,4,5). Our results represent the first evidence for a role for Sen1 in pre-mRNA metabolism. The spectrum of Sen1 mutant phenotypes is similar to those associated with several factors involved in nucleo-cytoplasmic transport of mRNA (6), suggesting that Sen1 may function in nuclear trafficking of pre-mRNA. Thus, our studies may help to identify factors and mechanisms controlling the nuclear fate of pre-mRNA and mRNA from transcription through transport.

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RNA PSEUDOKNOT FROM BEET WESTERN YELLOW VIRUS AT 1.9 Å RESOLUTION,

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The RNA pseudoknot is a very important tertiary motif widely found in virtually every class of RNA. In this motif, nucleotides from the loop region of a hairpin pair with a single stranded region outside the hairpin to form a quasi-continuous helix with loops crossing the major and minor groove. This unique structural motif is recognized by certain proteins and translational components to regulate translation on frameshifting and suppression. Furthermore and in particular, recent evidence shows that pseudoknots are potent inhibitors of the reverse transcriptase of HIV, the virus that causes AIDS. To date, no detailed information of the geometry of this motif at atomic resolution is available.

We were able to produce highly ordered single crystals of an RNA pseudoknot from Beet Western Yellow virus. This pseudoknot fragment is 28 nucleotides in length and involved in the translational frameshifting of open reading frames 2-3 to produce a fusion protein. The large amount of RNA used for crystallization was produced by the T7 RNA polymerase in vitro transcription system. Crystals of two different forms were obtained. One crystal lattice is trigonal, space group P3(1)21, unit cell a=30.84, b=30.84, c=144.55, α=90.0, β=90.0, γ=120.0, with 1 molecule in the assymetric unit. The diffraction limit is 1.9 Å, which is well beyond the resolution of crystals of comparable RNA molecules such as tRNA (2.8 Å) and ribozymes (2.6 Å). The other crystal form is cubic, I23, a=b=c=108.9, diffraction limit 2.8 Å. Structure analysis will proceed with Multiple Isomorphous Replacement and Molecular Replacement methods. Concurrently, we are performing Rnase mapping and chemical modification studies to probe the tertiary interactions in solution. The comparison of the pseudoknot structure in the two crystal forms along with the biochemical studies will allow us to gain sufficient knowledge of this motif.

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FROM TRANSCRIPT TO PROTEIN:
mRNA PROCESSING, TRANSPORT AND TRANSLATION

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