Instituto Juan March
de Estudios e Investigaciones

113 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by
EMBO EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Workshop on

Regulation of Messenger RNA Processing

Organized by

W. Keller, J. Ortín and J. Valcárcel

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Introduction

W. Keller, J. Ortín and J. Valcárcel

Gene expression starts with the synthesis of primary transcripts (pre-mRNAs) from DNA. Pre-mRNAs undergo extensive modifications in the nucleus before they can be transported to the cytoplasm and be translated into proteins. These modifications, collectively known as RNA processing, include addition of a methylated nucleotide at the 5' end (capping), a polyadenylate tail at the 3' end (polyadenylation), removal of internal sequences (splicing) and changes in the nucleotide sequence (editing).

Studies on the regulation of gene expression are essential to understand cell differentiation and development. The extent to which this regulation occurs at the level of messenger RNA processing, however, has only recently become fully recognized. Tissue-specific splicing, polyadenylation and editing are now phenomenologically well established as modes of gene regulation at the basis of key processes, from sex determination in Drosophila to maturation of the immune response, from fertilization to neural development. They are also at the origins of disease, from the control of blood cholesterol transport to viral pathogenesis to metastatic transformation of primary tumors. This wealth of biological implications has attracted the interest of many researchers to the molecular and cellular mechanisms underlying both the basic processes responsible for RNA modification and their key regulatory steps.

The Workshop sponsored by the Fundación Juan March and EMBO brought together leading world experts in the regulation of RNA processing to discuss common denominators between the control mechanisms operating in different processing steps, different organisms and biological phenomena. Many stimulating talks emphasized three main themes. First, the prevalence of mutual influences and intimate connections between different processing events, between these and transcription and nucleo-cytoplasmic transport, and between signal transduction pathways and processing factors. Second, many contributions illustrated how every processing step, even the apparently most simple, is accomplished by complex machines composed of multiple subunits. The roles of the different subunits are often enigmatic, presumably involved in regulation of the function of the complex. As an illustrative example, none of the multiple protein components of the factors

responsible for the endonucleolytic cleavage that occurs at the 3' end of most RNA polymerase II transcripts has the sequence features of an endonuclease. Will the catalytic activity be contributed by several subunits ? Will it be contributed by the RNA itself, perhaps conformationally activated by interactions within the complex ?

The third common thread was the realization that the different members of families of proteins, their isoforms and postranslational modifications can provide unique properties to regulatory assemblies, and therefore their precise composition and distribution of cis-acting elements can generate combinatorial complexity able to account for the design of gene expression in space and time.

Hopefully the participants left with renewed appreciation for the complexity and regulatory potential of RNA processing and stimulated to keep deciphering its pervading logic and beauty. There is no doubt that the peaceful and comfortable setting that the Fundación Juan March provided, together the helpfulness of its personnel, significantly contributed to achieve these goals.

W. Keller, J. Ortín and J. Valcárcel

Session 1
Chair: Nick Proudfoot

Analysis of nuclear structure and snRNP organisation *in vivo* using fluorescent protein fusions

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We are studying the functional organisation of the nucleus in mammalian cells. As a model system we have analysed the subnuclear organisation of the small nuclear ribonucleoproteins (snRNPs), which are subunits of spliceosomes. Splicing snRNPs show a complex "speckled" localisation pattern and associate with several distinct subnuclear structures, including perichromatin fibrils, clusters of interchromatin granules and Cajal bodies (also known as coiled bodies). To study the dynamic organisation of snRNPs *in vivo*, we have made fusions of the core snRNP Sm proteins to either the Green, Yellow or Cyan Fluorescent proteins (GFP, YFP & CFP). We have generated stable HeLa and MCF7 cell lines that express FP-tagged snRNP proteins. We have also made stable cell lines that express FP-tagged Cajal body and nucleolar proteins and non-snRNP protein splicing factors. FP-tagged snRNP proteins assemble into snRNP particles and adopt an identical *in vivo* localisation pattern to endogenous snRNPs. However, we find that newly assembled snRNPs concentrate in Cajal bodies prior to interchromatin granule speckles when they are first imported into the nucleus. A similar result is obtained by heterokaryon assays or by transient expression of FP-tagged snRNP proteins. In contrast, snRNPs reimported into the nucleus after mitosis immediately adopt a speckled pattern. The data indicate that snRNP maturation likely involves a sequential pathway of snRNP movement in the nucleus between distinct subnuclear domains. Our most recent results indicate that the pathway of snRNP movement from Cajal bodies to speckles may be obligatory.

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Cotranscriptional editing in paramyxoviruses

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The site-specific insertion of preferentially two G residues in a fraction of the phosphoprotein (P) gene transcipts of simian virus 5 (SV5) was discovered in the laboratory of R.A. Lamb (1); only the modified transcipts allow ribosomes, which on canonical transcripts produce a relatively short protein called V, to read further on in a different reading frame, thus yielding full length P protein. Independently, in our laboratory nearly half of the P gene transcripts of measles virus (MV) were revealed to carry one additional G residue (2); in case of this virus the unmodified P transcripts encode the full length P protein whereas the modification leads to formation of a V protein. Similar to the V of SV5 (and predicted V proteins of other paramyxoviruses) the MV V contains a relatively short C-terminal domain distinguished by a series of cysteine residues conserved in all these viruses. We termed the phenomenon of site-specific G insertions as cotranscriptional editing, since it appeared to rely on the transcriptase pausing and stuttering at a particular stretch of U and C residues, somehow analogous to the reiterative slippage of the polymerase on a short series of U residues at the end of each transcription unit of the genomic template, mediating the attachment of more than 100 A residues at the 3' termini of the transcripts. This notion was corroborated primarily by the group of D. Kolakofsky (3). Most representatives of the rubulavirus genus follow the pattern of SV5 whereas those of the genera respirovirus (e.g. Sendai virus (SeV), parainfluenza viruses (PIVs)), and morbillivirus (e.g. the MV related canine distemper virus (CDV) and rinderpest virus (RPV)) edit analogous to MV, although in some cases the incorporation of Gs is not strongly biased to the insertion of only a single residue; e.g. PIV3 forms P gene transcripts with one to six Gs added at almost equal frequency, and in this case protein variants called W arise on transcripts containing two or five inserted Gs (4).

The genomes (and antigenomes) of paramyxoviruses are always associated with nucleocapsid (N) molecules to form very tight RNP structures; analysis of SeV RNPs suggested that for every six nucleotides one N molecule is required (5), and that molecules of the multifunctional P and the large polymerase L are more loosely bound. The strictly stoichiometric association of N molecules to genome-type RNA was dramatically confirmed by the finding of Calain and Roux (6) that copy-back type defective interfering (DI) RNAs of SeV were replicated efficiently only when their total number of nucleotides was a multiple of six; this requirement was termed as "rule of six", which suggested that nascent genomic and antigenomic RNAs have to be consecutively covered from the 5' end by N molecules, with no "dangling" nucleotides allowed at the 3' termini. Later experiments revealed that also within the genomic and the antigenomic promoter distinct nucleotide hexamers must occur in specific spacial arrangements (7).

To test whether it also matters how the nucleotides of internal cis-acting elements such as the editing and polyadenylation sites are contacted by N molecules, we generated mutant MVs containing short insertions and deletions in the 3' terminal nontranslated regions of the N and H genes, thus shifting the large intervening genome section by -3, -2, -1, +1 and +2

nucleotides, respectively relative to the normal phase while preserving the total genome length. All the mutants, which propagated slowly in comparison to the standard virus, showed altered editing behaviours: the levels of P gene mRNAs with more that one G inserted were increased as shown by limited primer extension experiments. Northern blot analyses revealed that in some of these mutants the readthrough transcription into the next transcription unit is considerably increased in two of the four phase-shifted gene boundaries. Our results suggest that it is relevant in which phase N molecules contact editing and gene boundary region nucleotides; the RNP structure appears to remain intact at least quite close to the passing polymerase, and it is conceivable that some contacts between N molecules and the nucleotides of the template persist even at the regions active in transcription. Our results also document the high accuracy of RNP formation, and again confirm that the term „cotranscriptional editing" is justified.

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Role of PTB and CUG-BP isoforms in smooth muscle alternative splicing

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The pre-mRNAs of α-tropomyosin (TM) and α-actinin (Act) have pairs of mutually exclusive exons that are spliced with smooth muscle (SM) specificity. Both systems appear to involve regulation by members of the polypyrimidine tract binding protein (PTB) and CUG-binding protein families. However, the roles of the known members of these protein families appear to differ between the two systems.

In the better characterized TM system, the strong default exon 3 is repressed in SM cells. Repression involves *cis*-acting elements consisting of binding sites for PTB, and also CUG repeats. A number of lines of evidence have pointed to PTB as a repressor of TM exon 3[1-4]. However, we now find that PTB1 — the isoform used in most experiments — antagonizes regulated skipping of TM exon 3 when overexpressed in transfected SM cells. PTB exists in three common isoforms — 1, 2 and 4 — which differ by an additional 19 or 26 amino acid insert between RRMs 2 and 3 in PTB 2 and 4 respectively. Overexpression of the PTB 4 isoform had the opposite effect to PTB1; it enhanced TM exon skipping. PTB 2 had an intermediate effect. The same hierarchy for inducing exon skipping — PTB 4 > 2> 1 — was also found to hold in HeLa nuclear extracts *in vitro*. Parallel UV cross-linking showed that the recombinant proteins bound to the pre-mRNA and displaced the endogenous HeLa PTB equally well. Thus, the differential effects of the PTB isoforms appear to result from different inherent abilities to inhibit exon 3 splicing once bound to the RNA. The PTB isoforms were equally effective in inducing skipping of both the Act exons. PTB crosslinking to Act RNAs showed a good inverse correlation with the efficiency of splicing of the Act SM exon. However, binding of U2AF$_{65}$ was unaffected. This suggests that the mechanism of action of PTB in this case does not involve simple binding competition with U2AF.

We investigated the expression of PTB isoforms in primary rat aorta SM cells. These are initially highly differentiated, but rapidly dedifferentiate in culture with an accompanying switch in various alternative splicing patterns, including TM and Act. The ratio of PTB isoforms remained constant during this transition, and the overall levels of PTB appeared to increase. Thus, neither the PTB expression levels nor isoform composition appear to correlate with the TM regulated splicing patterns during dedifferentiation. However, a protein highly homologous to PTB was highly expressed in the differentiated primary cells; its levels decreased in parallel with loss of TM and Act regulated splicing, and with the increase in the levels of PTB. The sequence of the central section of this homologue, as revealed by RT-PCR, reveals that it arises from a novel PTB homologue gene, distinct from the neuronal nPTB/brPTB[5] and the hematopoietic Rod1[6] homologues. The novel homologue contains a PTB 4-like insert and an additional predicted flexible peptide linker between RRMs 3 and 4. It is not strictly restricted to SM cells but it is present at high levels in a number of differentiated SM tissues. This PTB homologue could be responsible, at least in part, for the high levels of repression of TM exon 3 in differentiated SM cells.

Both TM and actinin regulated exons are associated with clusters of CUG repeats. In collaboration with Tom Cooper (Baylor College of Medicine) we have tested the activities of three CUG-binding proteins[7] upon TM and Act splicing. None of the tested proteins appeared to be involved in regulation of TM exon 3 — their overexpression reduced exon 3 skipping, even though the CUG-elements mediate exon skipping. However, we found that both CUG-BP1 and Etr3 had opposite and independent effects upon selection of the two mutually exclusive Act exons. They promoted inclusion of the SM exon, but skipping of the NM exon. The third CUG-BP, (CELF4), had a different effect, promoting skipping of both the NM and SM exons. We also demonstrated the activation of SM exon and inhibition of NM splicing by Etr3 *in vitro*. Moreover, it appears that the activation of SM exon splicing by Etr3 involves displacement of the repressive PTB.

Overall, our data illustrate that control of TM and Actinin splicing is affected differentially by various members of the PTB and CUG-BP families. The precise combinations of these family members, which are derived by both alternative splicing and expression of multigene families, are likely to determine specific alternative splicing outcomes. In the future it will be of importance to determine the complement of these proteins — potentially including novel family members — that are present in fully differentiated SM cells.

Work in our laboratory is supported by the Wellcome Trust.

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A web of regulatory interactions among 3'UTR regulators controls diverse events in the *C. elegans* germline

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In *C. elegans*, the germ cell precursors proliferate mitotically at one end of the germline tube and enter the meiotic cell cycle as they begin to mature. In hermaphrodites, the first cells to undergo gametogenesis adopt a sperm fate, while subsequent cells become oocytes. We have found that each of these steps in germline regulation relies on specific putative 3'UTR regulators, and that these proteins physically interact.

C. elegans FBF and *Drosophila* Pumilio and prototype members of the Puf family of RNA binding proteins (1). The *C. elegans* genome encodes ten PUF proteins, of largely unknown function. The hermaphrodite switch from spermatogenesis to oogenesis requires two *fbf* genes and three Nanos genes, *nos-1 – nos-3*. Moreover, germline survival similarly requires both Puf and Nos proteins (3,7). Judged by two-hybrid and GST-pulldown experiments, FBF binds NOS-3 (3) and two other PUF proteins, PUF-6 and PUF-7, bind specifically to NOS-1 and NOS-2. Therefore, distinct PUF/NOS partner pairs are involved in germline fate regulation.

In addition to its interactions with NOS-3, FBF also binds to CPB-1, a *C. elegans* homolog of the *Xenopus* 3'UTR regulator called CPEB (2). In many organisms, CPEB homologs are expressed in the female germline and control various steps in oogenesis. In contrast, we find that CPB-1 is dispensable for oogenesis in *C. elegans*, but is required for spermatogenesis: in its absence, spermatogenesis arrests prematurely – probably at the primary spermatocyte stage. CPB-1 protein is present just prior to overt sperm differentiation, consistent with its function. We show that although FBF-deficient animals produce mature sperm, those sperm generate few viable embryos. We suggest that CPB-1 and FBF collaborate to execute a late step in spermatogenesis. The presence of CPEB transcripts in the testes of other species suggests that a role in spermatogenesis may be common, albeit largely unexplored to date.

A second CPEB homolog in *C. elegans* is required for the specification of a germ cell as a sperm rather than an oocyte (1). This homolog is called *fog-1*. In animals lacking *fog-1* activity, any cells that would normally differentiate as sperm become fully functional oocytes instead. In wild-type animals, the *fog-1* gene is regulated to produce different mRNAs during spermatogenesis and oogenesis. The shorter *fog-1* mRNAs that are associated with oogenesis encode an N-terminally truncated FOG-1 protein. Thus, two CPEB homologs are required for spermatogenesis, and are dispensable for oogenesis.

Finally, both FBF and FOG-1 are required for germ cell mitoses. In deletion mutants lacking FBF, the germline is smaller and all cells differentiate as sperm. In mutants lacking both FBF and *fog-1*, the germline precursor cells divide only once or twice. Thus these two genes are required in redundant fashion for germline mitoses.

In sum, these data suggest that a wide spectrum of events in the *C. elegans* germline rely on 3'UTR-based regulators and their interactions. They suggest that combinatorial interactions among regulators control distinct mRNAs and biological outcomes.

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Negative strand virus RNA polymerase stuttering

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Negative strand virus RNA polymerases carry out RNA synthesis in two modes, transcription (mRNA synthesis) and replication. The essential difference between these two modes of viral RNA synthesis is that during transcription vRNAP responds to template cis-acting signals which specify vRNAP stuttering (or pseudo-templated synthesis), both to "edit" the P gene mRNA by G insertions, and to add a poly(A) tail to each mRNA (and to concomitantly terminate each mRNA chain).

vRNAP stuttering requires a template homopolymer stretch (which can be as short as 3 nucleotides for mRNA editing and 4 nucleotides for poly(A) tail formation), as well as a heteropolymeric controlling sequence which maps just upstream of the homopolymer run. The mechanism by which this controlling sequence acts can be investigated by reverse genetics. These results will be discussed with reference to the recently elucidated structures of cellular RNAPs.

Session 2
Chair: Iain Mattaj

3'-end processing/polyadenylation of messenger RNA precursors (pre-mRNAs) and editing of pre-mRNAs and tRNAs

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We are studying the 3'-end processing pre-mRNAs in a mammalian system and in yeast (*S. cerevisiae)*. The reaction comprises two simple chemical steps, endonucleolytic cleavage of the RNA substrate and addition of a poly(A) tail to the upstream cleavage product. Nevertheless, the process requires a multicomponent machinery of remarkable complexity. Cleavage is preceded by the assembly of five protein factors upstream and downstream of the cleavage site. Most of the trans-acting factors consist of multiple polypeptides, some of which bind to the RNA and others form protein-protein contacts between the factors. Most of these factors have been purified and cloned, and their properties will be discussed.

As a first step towards getting a high-resolution picture of the polyadenylation machinery, we have determined the crystal structure of mammalian poly(A) polymerase complexed to 3'-dATP in the active site. The structure provides insights to the mechanism of polyadenylation and to the substrate specificity of the enzyme.

In yeast, we have identified and cloned the complete set of the 3'-end processing components, the products of fifteen essential genes. Results of mutant analyses, sequence features shared with their mammalian homologues, reconstitution of functional factors from recombinant subunits and their interactions within the complex and with the RNA substrate will be presented.

Finally, we will report on a comparative study of RNA-specific adenosine deaminases that catalyze the conversion of adenosine residues to inosine. These enzymes belong to a large family of proteins that are related in their catalytic domain, but differ in their substrate specificities. ADARs can selectively edit a number of adenosine nucleotides in pre-mRNAs coding for synaptic receptors in the central nervous system whereas ADATs modify specific single adenosines in different tRNA molecules. The sequence relationships of the different enzymes suggest a possible scheme for the evolution of RNA editing enzymes.

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Control of gene expression by influenza virus NS1 protein

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Influenza virus NS1 protein is an RNA-binding protein whose expression in mammalian cells alters various cellular post-transcriptional processes. Thus, it inhibits pre-mRNA polyadenylation and splicing and mRNA nucleo-cytoplasmic transport. In addition, NS1protein stimulates translation of viral mRNAs. To investigate the mechanisms responsible for these effects, we have looked for cellular proteins able to interact with NS1 protein by co-immunoprecipitation experiments and two-hybrid screening in yeast.

We have found that NS1 protein co-immunoprecipitates with translation initiation factor 4GI, the large subunit of eIF4F, in cotransfected cells and in influenza virus-infected cells. Purified NS1 protein could pull-down 4GI protein but not the 4E subunit of eIF4F. Mapping studies using 4GI deletion mutants indicated that the NS1 binding domain is located between positions 157 and 550 in 4GI protein, a region where no other component of the translation machinery is known to interact. On the other hand, the N-terminal 113 amino acids of NS1 protein are sufficient for interaction with 4GI protein. Such a deleted protein had been previously shown to enhance translation of influenza virus mRNAs *in vivo*. Collectively, these data suggest that NS1 protein recruits 4GI factor specifically to viral mRNAs and allows for their preferential translation in infected cells.

As a result of a two-hybrid screen with NS1protein as a bait, a human cDNA clone was identified capable of coding for a protein with high homology to the Staufen protein from *D.melanogaster* (dmStaufen). The encoded protein (hStaufen) contained 4 dsRNA binding domains with 38% identity to those of dmStaufen, including identity at all residues involved in RNA binding. A recombinant protein containing all dsRNA binding domains, expressed in *E. coli* as a His-tagged polypeptide, showed dsRNA binding activity *in vitro*. Using a specific antibody, a main form of the hStaufen protein could be detected in human cells, with apparent molecular mass of 60-65 kDa. The intracellular localization of hStaufen protein was investigated by immunofluorescence, using a series of markers for the cell compartments. Co-localization was observed with rough endoplasmic reticulum, but not with endosomes, cytoskeleton or Golgi apparatus. Furthermore, sedimentation analyses indicated that hStaufen protein associates with polysomes. The interaction of hStaufen with NS1 protein was studied both *invivo* and *in vitro*. Both proteins could be co-immunoprecipitated from influenza virus-infected cells, when co-expressed in cultured cells or when mixed *in vitro*. Furthermore, hStaufen and NS1proteins co-fractionate in the polysomes of influenza virus-infected cells.

To gain information about the role of hStaufen protein in cultured human cells and the possible implication of its association to NS1 in the influenza virus infection, we set out to identify RNAs and proteins that associate to hStaufen in human cells. To that aim we have expressed a tagged hStaufen protein by transfection. Such a tagged protein localized intracellularly in a manner indistinguishable from endogenous wt hStaufen. The purification of this recombinant hStaufen and the identification of the attached RNAs and proteins is in progress.

Mechanism of kinetoplastid RNA editing

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Kinetoplastid protozoa edit many of their mitochondrial mRNAs by the insertion and deletion of uridylates (Us). The editing produces functional mRNAs and the regulation of editing controls the alternation between glycolytic and cytochrome mediated oxidative phosphorylation modes of energy production during the life cycle of these organisms. The editing is accomplished by a series of enzymatic steps that are catalyzed by a macromolecular complex. These enzymatic steps include endoribonucleolytic cleavage of the pre-edited mRNA (pre-mRNA) and addition or removal of uridylates at the 3' end of the 5' cleavage product followed by its ligation with the 3' pre-mRNA fragment. The edited sequence is specified by guide RNAs (gRNAs). We explored the contribution of each catalytic step to the specificity of the edited sequence using partially purified editing complexes. The endoribonuclease selects (cleaves) the editing site and leaves the phosphate at the 5' end of the 3' cleavage product. This cleavage usually occurs at the first single-stranded site 5' to the "anchor" duplex between the pre-mRNA and gRNA. However, the cleavage does not invariably occur at such a site indicating other substrate requirements for this activity. This incomplete specificity may explain the character of partially edited pre-mRNAs. The U addition activity, or 3' terminal uridylate transferase (3'TUTase) is specific for uridylates. It shows a strong preference for UTP, which is greatly enhanced when more than one addition is specified by the gRNA. The number of Us that are added is affected by their ability to basepair with corresponding nucleotides in the gRNA and the character of the substrates. The U deletion activity is also specific for Us and hence is a 3' exouridylylase. The number of Us that are removed is affected by basepairing with gRNA suggesting that the 3' exoUase utilizes a single stranded substrate. The presence of both activities in the editing complex suggests that they may act together to produce a 3' fragment with the number of US specified by the gRNA. The RNA ligase activity is dramatically enhanced by a "splinting" gRNA. In addition, 5' and 3' pre-mRNA fragments with "gaps" or "overhanging" Us are ligated but fragments lacking gaps or overhangs are ligated more efficiently. Hence, each of the activities in the editing complex acts in concert to produce precisely edited RNA.

The editing complex was purified by successive fractionation by two ion exchange columns, a size exclusion column and glycerol gradient sedimentation using deletion editing activity to monitor purification. Insertion editing and endoribonuclease, TUTase, 3' exoUase, and RNA ligase activities were also monitored during the fractionation. The most purified fraction had an apparent mass of 1.6 million KDa, contained 20 major proteins as well as all of the editing activities. Mass spectroscopic analysis of the proteins identified the genes for 15 of the proteins; 11 are novel, one is an

RNA helicase that was previously identified as a candidate component of the editing complex and three are likely contaminants. The identity of two of the genes was confirmed by N-terminal sequencing or by recognition of recombinant protein by a monoclonal antibody prepared against the native complex. Three of four monoclonal antibodies that recognize proteins in the purified complex immunoprecipitate the editing activities. In addition, the protein profile of the immunoprecipitated complex is very similar to that of the biochemically purified complex. Studies to explore the functions of the components of the complex are in progress.

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Recursive splicing and genetic analysis of splice site regulation in *Ultrabithorax*

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(A) Recursive Splicing in Large Introns: Very large introns are found in multiple genes that play important roles in metazoan development and human disease, but little is known about mechanisms that may facilitate their accurate and efficient processing. Potential problems with cryptic splice sites and inappropriate inclusion or exclusion of internal exons may be minimized if smaller subfragments of such introns can be removed as they are transcribed. In principle, this could be accomplished by "recursive splicing" using internal elements that function initially as 3' splice sites but regenerate 5' splice sites when joined to an upstream exon. We have shown that two cassette exons in the *Ultrabithorax (Ubx)* gene of Drosophila serve in this way to subdivide a 77 kb intron even in tissues and stages where they are excluded from the mRNAs (ref. 1). The 5' ends of these exons regenerate 5' splice sites, leading to their regulated removal after constitutive splicing to the upstream exon. Analysis of sequence databases reveals that large introns in Drosophila and mammals contain multiple potential ratchetting points (RPs) that are not part of known cassette exons but might function by the same recursive splicing mechanism. We have analyzed 7 potential RPs in three different genes of Drosophila and have demonstrated the existence of the predicted ratchetting intermediates, whereas no associated exons are detectable in mRNAs. We have focused on a potential RP in the middle of a 52 kb intron in *Ubx* and have demonstrated that it regenerates a strong 5'ss after it is joined to the upstream exon. Furthermore, splice site competition experiments using wild type and mutant transgenes indicate that most or all *Ubx* transcripts are processed using this element as an invisible ratchetting point. RPs raise new questions about the mechanisms that define exons, promote accurate recognition of splice sites, regulate splicing and contribute to evolution of gene structure. Sequence comparisons suggest that a stereotyped arrangement of small downstream pseudo-exons and pseudo-introns may contribute to recognition of the RPs and may account for their sequential activity as 3' and 5' splice sites.

(B) Regulation of 5' splice site competition in *Ultrabithorax*. We are using *Ultrabithorax* as a model for complex developmental regulation of alternative splicing. As described above, three major *Ubx* isoforms are generated by recursive splicing of two cassette exons (mI and mII) using 5' splice sites regenerated at their junctions with the upstream exon (ref. 1). The structures of the isoforms and their complex patterns of developmental regulation have been conserved over at least 100 million years of insect evolution (ref. 2). Our current studies focus on the control of exon mI. We have used phylogenetic comparisons, in vivo and in vitro mutagenesis, transgenesis and biochemical analysis of RNA-protein interactions to identify cis-acting elements and trans-acting factors involved in regulation (ref. 1-4,6). These studies reveal that the complex developmental pattern of mI inclusion is generated by

multiple regulatory inputs. Use of the regenerated 5' splice site is promoted and repressed through overlapping elements (R and H) within mI. These elements appear to mediate the action of proteins related to mammalian SRp20 (Drosophila Rbp1, which binds element R) and hnRNP A1 (Drosophila hrp48, which binds element H). Use of the competing downstream 5' splice site is favored by a third element (C, located near the center of mI) that resembles a selex-defined binding site for the SRp55 homolog B52 but probably does not function in this capacity (ref. 1,4). Use of the downstream 5' splice site is also favored by a specific interaction with the next exon (mII) across the intervening intron (ref. 1,3).

Parallels with the regulation of *Sxl* and *P* element splicing suggest a common mechanism for 5' splice site repression involving recruitment of U1 snRNP into a non-productive complex, with variations that probably account for the differences in developmental specificity and quantitative features. Like autoregulation of *Sxl* splicing, repression of the regenerated 5' splice site for mI requires the function of SNF (the homolog of both U1A and U2B"), and the novel proteins VIR and FL2D (ref. 4,5; *Sxl* data reviewed in 6). Expanding on proposals by Helen Salz and Paul Schedl, we suggest that these proteins form part of a complex that recruits U1 snRNP but prevents it from participating in spliceosome assembly. In recent screens we have identified two new genes (*Hel25E* and *EUb-28*) that are also required for splicing regulation of both *Ubx* and *Sxl* (ref. 5,6). *Hel25E* encodes a Drosophila homolog of mammalian UAP-56, previously proposed to interact with U2AF to aid recruitment of U2 snRNP. We are working to elucidate its role in *Ubx* splicing and to identify the product of *EUb-28*, which we isolated as an enhancer of the mI-exclusion phenotype caused by a gain-of-function mutation in B52/SRp55. SXL protein, which interacts with SNF and with cis-acting elements on the *Sxl* RNA, is not required for *Ubx* splicing regulation; instead, a targetting function similar to that of SXL appears to be performed by hrp48. hrp48-binds to element H, which overlaps the regulated 5' splice site in an arrangement similar to that of pseudo-5' splice sites F1 and F2 within the splicing regulatory signal in *P* element RNA (reviewed in ref. 6; in the regulatory complex F1 contacts U1 snRNP, whereas F2 contacts hrp48). Reduction of hrp48 expression increases the frequency of mI resplicing, whereas overexpression of hrp48 reduces the frequency of resplicing. Four additional genetic loci required to prevent resplicing of mI have been identified. Analysis of deletions defines two loci required for removal of mI; one of these corresponds to the location of *Rbp1*, in which mutations have not yet been identified.

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mRNA processing and the integration of nuclear events

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Evidence has accumulated in recent years supporting the existence of direct links between mRNA transcription and the related RNA processing events, capping splicing and polyadenylation. In all three reactions, RNA polymerase II, and specifically the C-terminal domain of its largest subunit, plays a key role. I will describe here experiments that extend further the link between transcription and mRNA processing, and which provide genetic and biochemical evidence for an evolutionarily conserved interaction between the transcriptional and polyadenylation machineries. I will also describe data supporting more unexpected connections. For example, we have found that polyadenylation may be linked to DNA damage, such that treatment of cells with DNA damage-inducing agents strongly but transiently inhibits 3' processing in cell extracts. We show that this reflects in inhibition interaction between the BRCA1 tumor suppressor-associated protein BARD1 and CstF. A tumor-associated BARD1 mutant protein is inactive, suggesting a link between tumor suppression and DNA repair-induced inhibition of polyadenylation. Finally, our previous work has established a role for control of polyadenylation during the cell cycle, and I will describe experiments detailing cell cycle-related regulation of poly(A) polymerase.

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Session 3
Chair: Angus I. Lamond

Roles of Ran in interphase and mitosis

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The RanGTPase plays a critical role in many forms of nucleocytoplasmic transport. Ran provides the energy required for these forms of transport as well as determining the direction in which transport proceeds (1, 2). Among the simple forms of transport that require Ran is the nuclear export of tRNA, mediated by the Xpo-t nuclear export receptor (3). A much more complex example is export of the spliceosomal U snRNAs. Here not only an export receptor (Xpo-1) is involved but also two different adaptors, CBC and PHAX (4).

Ran's function is however not confined to nuclear transport. RanGTP is also required during mitosis to induce spindle assembly (5). A second role of RAN during mitosis is in the re-assembly of the nuclear envelope at anaphase (6).

The various functions of Ran, and their molecular mechanisms, will be compared.

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New C to U RNA editing enzymes

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C to U editing of apolipoprotein (apo) B mRNA is mediated by tissue specific RNA binding cytidine deaminase APOBEC1. APOBEC1 is structurally homologous to *Escherichia coli* cytidine deaminase (ECCDA) but has evolved specific features required for RNA substrate binding and editing. A signature sequence for APOBEC1 has been used to identify other members of this family. One of these genes, designated APOBEC-2 is found on chromosome 6. Another gene corresponds to the AID gene, which is located adjacent to APOBEC-1 and chromosome 12. Seven of these genes, or pseudogenes (designated APOBEC-3A to G) are tandomly arrayed on chromosome 22. Four of the genes correspond to the amino terminal two thirds of APOBEC-1 and have four exons. The other three genes are directly duplicated and have seven or eight exons. This locus is not present in rodents. It is apparently an anthropoid specific expansion of the APOBEC family. The conclusion that these new genes are new members of the APOBEC family comes from similarity in amino acid sequence with APOBEC1, conserved intron/exon organisation, tissue specific expression, homodimerization like APOBEC-1 and zinc and RNA binding. One of these genes is a pseudogene and another is possibly an expressed pseudogene that produces a dysfunctional protein. Tissue specific expression of these genes includes high level expression in a variety of tumour cell lines derived from solid cell and haematological tumours. The high level expression of these genes in tumour cell lines, along with other evidence suggesting that APOBEC-1 is an oncogene, suggests a role for these enzymes in growth or cell cycle control and a possible role in neoplasia.

Control of *Drosophila* P element transposition through RNA processing

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The P transposable element third intron (IVS3) is spliced only in *Drosophila* germline cells to produce mRNA encoding the transposase protein. IVS3 is fully retained in somatic cells and the resulting mRNA is exported to the cytoplasm to encode a truncated protein that acts to repress transposition. One aspect of the regulation of this tissue-specific alternative splicing event involves inhibition of IVS3 splicing in somatic cells and requires a *cis*-acting negative regulatory sequence in the IVS3 5' exon, adjacent to the accurate IVS3 5' splice site. Work to date indicates that RNA-binding proteins in *Drosophila* somatic cell (Kc) nuclear extracts interact with this exonic regulatory element and these RNA-protein intereactions prevent binding of U1 snRNP to the accurate IVS3 5' splice site resulting in a block to the early steps in spliceosome assembly.

Two components that interact with the IVS3 5' exon negative regulatory element been previously been purified from Kc nuclear extracts: the *Drosophila* hnRNP protein hrp48 and the P-element somatic inhibitor protein (PSI). Both of these factors bind to the 5' exon negative regulatory sequence and are required for somatic inhibition of IVS3 splicing *in vitro* and *in vivo*. Hrp48 is similar to mammalian hnRNPA1, is expressed in both the soma and the germline and interacts with a sequence in the 5' exon, called F2, that resembles a 5' splice site. PSI is abundant only in somatic cells suggesting that it acts as a tissue-specific alternative splicing factor. Ectopic overexpression of PSI in the female germline reduces IVS3 splicing *in vivo*. U1 snRNP may also be a component of the somatic inhibitory activity as it binds to another 5' splice site-like sequence, called F1, present in the IVS3 5' exon regulatory sequence. Mutations in the upstream U1 binding site (F1) or in the hrp48 bindng site (F2) relieve inhibition and allow IVS3 splicing *in vitro* and *in vivo*.

The PSI protein contains four KH-type RNA binding domains and two C-terminal glutamine-rich repeats (A and B motifs) that mediate protein-protein interactions with the RS domain of *Drosophila* U1-70K protein *in vitro*. This interaction is very specific and is completely abolished by point mutation of crucial amino acids in the A/B repeats that are conserved between PSI and its mammalian homologues, KSRP and FBP-1/3. *In vivo*, we used the UAS/Gal4 system to ectopically express PSI transgenes in the germline and demonstrated that the A/B motifs are involved in splicing inhibition of IVS3 reporter constructs. Furthermore, while overexpression of PSI early in development leads to embryonic lethality, no phenotype was observed for the protein lacking the A/B motifs suggesting that these motifs may be involved in the splicing regulation of other cellular pre-mRNAs. To test this hypothesis, we took advantage of a line carrying a deletion mutation in the PSI gene that causes a 200-fold reduction in viability and can be rescued by a transgene bearing the PSI cDNA. However, only 80% of the expected flies were observed with a transgene deleted of the A/B motifs and these flies exhibited important developmental defects. Using the differential display technique (DD-PCR), a comparative study led to the identification of several mRNAs that were altered in the ΔA/B transgenic line.

In order to identify the binding sites for the KH domains of the PSI protein, an *in vitro* selection (SELEX) was performed using a random oligonucleotide RNA pool. Alignment of selected high affinity PSI binding sequences revealed a specific consensus motif that resembles the F2 pseudo-5' splice site in the IVS3 5' exon regulatory element. Additionally, a negative *in vitro* selection of PCR mutagenized P-element 5' exon regulatory element RNAs identified two U residues as important nucleotides for PSI binding and one of these residues is a nearly invariant nucleotide in the above consensus motif derived from the positive SELEX experiment. We are testing the functional activities of these SELEX sequences in the context of the P-element pre-mRNA and in heterologous splicing substrates. Preliminary data from UV cross-linking, splicing and competition experiments indicate a correlation between the binding affinities of PSI for the SELEX sequences, and their ability to modulate splicing of P-element IVS3 *in vitro*.

Finally, we have used RNA affinity chromotography to identify other RNA binding proteins that interact with the IVS3 5' exon negative regulatory element. UV photochemical crosslinking had previously identified a species of ~65kD that interacted with the P element 5' exon. Biochemical fractionation, peptide sequencing and cDNA cloning have identified two different polypeptides that bind P element 5' exon RNA. Characterization of these two proteins is currently underway.

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PTB antagonizes exon definition in FGF-R2 transcripts

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Here I will present two studies on the regulation of splicing, one is an example of natural regulation of alternative splicing and the other is a method of re-programming gene expression with applications in gene therapy and genomics. Alternative splicing is a major driving force for proteomic diversity. Alternative splicing of fibroblast growth factor receptor 2 (FGF-R2) transcripts involves the mutually exclusive usage of exons IIIb and IIIc to produce two different receptor isoforms with very different function. Appropriate splicing of exon IIIb in rat prostate cancer DT3 cells requires a previously described cis-element (ISAR: intronic splicing activator and repressor) which represses splicing of exon IIIc and activates splicing of exon IIIb. We have now identified two intronic elements that cause repression of exon IIIb splicing. Deletion of either one of these elements abrogates the requirement for ISAR in order for exon IIIb to be spliced in DT3 cells. The element are intronic splicing silencers (ISS) sequences upstream (ISSU) and downstream (ISSD) of exon IIIb. Both these elements contain putative binding sites for the polypyrimidine tract binding protein (PTB). In vitro crosslinking studies demonstrate that ISSU and ISSD bind PTB. Mutations within ISSU that abolish PTB binding in vitro alleviate splicing repression in vivo. Co-transfection of a PTB expression vector with a minigene containing exon IIIb and ISSU demonstrates a dose dependent PTB mediated repression of exon IIIb splicing. These data are the first results to demonstrate direct repression of splicing by PTB in vivo. We offer a model for the global repression of exon IIIb and its cell-type specific de-repression to explain the tissue specific expression of the IIIb isoform of FGF-R2. The spliceosome, a macromolecular enzyme, precisely and efficiently removes these introns in a process known as pre-messenger RNA splicing. This enzyme is also capable of recombining two RNAs in trans in a process known as spliceosome mediated RNA trans-splicing. We have previously demonstrated that spliceosome mediated RNA trans-splicing can target and reprogram pre-messenger RNAs in vitro and in vivo. To understand the mechanism of trans-splicing in greater detail and to explore its potential to repair mutations associated with genetic defects, an endogenous LacZ repair model was developed. We constructed intron-containing LacZ target genes capable of normal splicing. These genes contain nonsense stop codons and were defective in producing functional ß-galactosidase; therefore, they served as a model for loss of function in genetic diseases. [Pre]-trans-splicing RNA molecules were designed to bind specifically to the target and replace the defective exon through RNA trans-splicing. Trans-splicing between LacZ pre-mRNA targets and [pre]-trans-splicing RNA molecules was efficient and precise, producing _-galactosidase activity ~200-fold above the background levels in co-transfection assays and ~14-fold in stable cells expressing an integrated defective LacZ pre-mRNA target. A semi-quantitative assessment of RNA trans-splicing showed the level to be greater than 10% when targeting endogenous pre-mRNA. These results demonstrate the ability of spliceosome mediated RNA trans-splicing to repair both exogenous and endogenous pre-mRNA targets with the concomitant production of functional protein. These observations strongly suggest that this technology may have applications in the repair of mutations underlying many human genetic diseases.

Retroviral nuclear RNA export pathways

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Retroviral replication requires the expression of spliced and unspliced forms of a single initial genomic transcript. However, because the eukaryotic cell has mechanisms in place to prevent the nuclear export of incompletely spliced pre-mRNAs, retroviruses have had to evolve alternate mechanisms to export their incompletely spliced transcripts. At least two distinct RNA export pathways utilized by retroviruses have been defined. HIV-1 and other complex retroviruses encode a nuclear RNA export factor, of which the prototype is HIV-1 Rev, that recruits the Crm1 nuclear RNA export factor to a cis-acting RNA target termed the RRE. In contrast, some simple retroviruses encode a structured RNA target, termed a CTE, that in the case of MPMV has been shown to function as a direct target for a distinct cellular RNA export factor termed Tap. I will discuss our recent research into how Tap mediates MPMV nuclear RNA export and will also describe our progress in understanding the mechanism of action of a fully functional Crm1 dependent nuclear mRNA export factor that is encoded within a family of endogenous proviruses located at >100 copies per human genome.

Session 4
Chair: Marv Wickens

Masking and unmasking 3'UTRs in mouse oocytes

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Modulation of mRNA translation is a mechanism of control of gene expression that could play a particularly important role in cell types where a temporal or spatial uncoupling between transcription and protein synthesis is required. During gametogenesis, some of the changes in the pattern of gene expression that occur while the genome is undergoing meiosis rely on the activation of dormant mRNAs: in oocytes, for instance, a subset of mRNAs accumulates during the growth phase of the cells and remains, stable and untranslated, until meiotic maturation is triggered. Studies in oocytes from a variety of species have elucidated aspects of the mechanisms that are responsible for the translational control of such "maternal" mRNAs.

We have used the modulation of synthesis of the serine protease tissue-type plasminogen activator (tPA) as a paradigm of translational control in mouse oocytes. tPA mRNA is transcribed in growing primary oocytes, where it undergoes a partial removal of its poly(A) tail and accumulates, untranslated, in the cytoplasm of the cells. Following germinal vesicle breakdown, which signals resumption of meiosis (meiotic maturation), tPA mRNA undergoes extensive readenylation and is translated. The cis-acting determinants responsible for deadenylation and silencing of tPA mRNA in primary oocytes reside in a UA-rich "adenylation control element" (ACE) located in the 3'UTR of the transcript, upstream of the AAUAAA cleavage and polyadenylation signal. The ACE is masked from hybridisation with injected antisense oligonucleotides, presumably through the binding of an approximately 72 kDa protein. This masking appears critical for silencing of the mRNA, since injection of a competitor transcript containing the ACE triggers translation of tPA mRNA without resumption of meiosis or readenylation of the mRNA. During meiotic maturation, the 72 kDa protein is modified and released from the ACE region, which thus becomes partly unmasked. The specific readenylation process that occurs at this stage depends on the presence of both the ACE and the AAUAAA sequences. Readenylation appears critical for sustained translation of tPA mRNA, at a time when many other previously translated mRNAs are undergoing deadenylation and degradation.

Thus, masking and unmasking of a maternal mRNA appear critical to control its translation in mouse oocytes and, together with regulated polyadenylation, achieve the temporal modulation of gene expression that is required for gametogenesis. Such a translational control mechanism may operate also in cells other than oocytes. To investigate this possibility, we have prepared mice carrying an ubiquitous promoter-driven transgene that encodes a reporter protein (GFP) and contains the ACE sequence in its 3'UTR. Analysis of oocytes from such transgenic mice confirms the validity of the approach, in that reporter expression is regulated just like that of tPA. Ongoing studies are aimed at identifying cell types in which a discrepancy between levels of the reporter encoded mRNA and expression of the reporter protein may occur.

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Regulation of RNA splicing during an adenovirus infection

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Adenovirus gene expression is to a large extent regulated at the level of alternative pre-mRNA splicing (reviewed in Imperiale et al., 1995). We are using the major late region 1 (L1) as a model substrate to study the virus-induced events regulating RNA splice site choice. L1 represents an example of an alternatively spliced gene where the last intron is spliced using a common 5' splice site and two competing 3' splice sites, generating two predominant cytoplasmic L1 mRNAs; the 52,55K (proximal 3' splice site) and the IIIa (distal 3' splice site) mRNAs, respectively. The 52,55K mRNA is produced both early and late after infection, whereas the IIIa mRNA is produced exclusively late. Activation of IIIa splicing late during infection results from an enhanced efficiency of IIIa 3' splice site recognition, combined with a virus-induced repression of 52,55K splicing (Kreivi & Akusjärvi, 1994). Our recent work has demonstrated that IIIa splicing is tightly regulated by two *cis*-acting viral elements: the IIIa repressor element (3RE) (Kanopka et al., 1996), and the IIIa virus-infection dependent splicing enhancer (3VDE) (Mühlemann et al., 2000). The newly discovered 3VDE appear to make the most significant contribution to activated IIIa splicing late during infection. However, very little is known about its mechanism of action. In contrast, we know a great deal about the function of the 3RE. Thus, the 3RE, which is located immediately upstream of the IIIa branch site, block IIIa splicing by binding the SR family of splicing factors (Kanopka et al., 96). Late during an adenovirus infection the SR proteins become functionally inactivated, as IIIa splicing repressor proteins, by a virus-induced dephosphorylation (Kanopka et al., 1998). This post-translational modification reduces their capacity to bind to the 3RE, and as a consequence alleviates their repressive effect on IIIa splicing. We have further shown that the virus-encoded E4-ORF4 protein can induce SR protein dephosphorylation, and has the capacity to activate IIIa pre-mRNA splicing, both *in vitro* and in transiently transfected cells (Kanopka, et al., 1998). Importantly E4-ORF4 binds the cellular protein phosphatase 2A (PP2A) and has been proposed to induce dephosphorylation of regulatory factors involved in both transcription and RNA splicing (Bondesson et al., 1996; Kanopka et al., 1998).

Here we show that the 3RE is both necessary and sufficient for E4-ORF4 induced activation of splicing. Furthermore, we show that E4-ORF4 interacts with only a subset of HeLa SR proteins; ASF/SF2 and SRp30c, respectively. In fact, E4-ORF4 interaction with the essential SR protein, ASF/SF2 correlates with E4-ORF4 activation of IIIa splicing. Assuming that SR protein inactivation is important for virus replication one would predict that overexpression of a SR protein during lytic growth should interfere with viral multiplication by shifting the balance of functional-non functional SR proteins towards the active form. This hypothesis was tested by analyzing the phenotype of a recombinant adenovirus expressing ASF/SF2 under the transcriptional control of a regulated promoter (Molin & Akusjärvi, 2000). The results show that, as predicted, ASF/SF2 blocks the early to late shift in viral mRNA expression. Furthermore, ASF/SF2 overexpression blocked viral DNA replication, inhibited major late mRNA accumulation, and reduced new virus particle

formation. Collectively our results provide additional support for the hypothesis that viral control of SR protein activity is important for efficient virus growth.

We previously suggested that ASF/SF2 binding to the 3RE blocked IIIa splicing by sterically interfering with U2 snRNP recruitment to the IIIa branch site (Kanopka et al., 1996). However, our more recent studies, using MS2-ASF/SF2 hybrid proteins, show that ASF/SF2 encode for a specific repressor domain. Thus, the ASF/SF2 RS domain functions as a splicing enhancer domain whereas the ASF/SF2 RBD2 is sufficient for splicing repression. Interestingly, the activity of RBD2 and the RS domain in splicing regulation is position dependent, and opposite. Thus, RBD2 inhibit splicing when tethered to the intron, but has no effect when binding to the second exon, whereas the RS domain activates splicing when tethered to the second exon but has no effect at an intronic position.

History has taught us that viruses typically target the same key regulatory pathways to take control of the biosynthetic machinery of the host cell. We have therefore initiated a study to determine whether other viruses also encode for proteins that regulate SR protein activity and RNA splicing. Our results show that like adenovirus, vaccinia virus functionally inactivates SR proteins as splicing enhancer or repressor proteins by a virus-induced dephosphorylation. We further show that the vaccinia virus encoded H1 phosphatase induces SR protein dephosphorylation. Also, the polyoma virus small T antigen (py-sT) activates IIIa pre-mRNA splicing both *in vitro* and *in vivo*. Py-sT, like E4-ORF4, interacts with PP2A. However, much to our surprise, py-sT activates IIIa pre-mRNA splicing through the 3VDE, in a catalytic reaction that appears to make IIIa splicing independent of both PP1 and PP2A.

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The multifunctional Herpes simplex virus ICP 27 protein

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Herpes simplex virus (HSV) is a double stranded nuclear replicating virus. Two HSV regulatory proteins, expressed during the immediate early phase of replication, are essential for viral growth and one of these, ICP27 (IE63), is the only HSV immediate early gene with a counterpart in all sequenced herpesviruses of mammalian and avian origin. Studies of ICP27, which may perform similar functions throughout the Herpesviridae, are important to key questions about herpes biology and the development of effective antivirals.

ICP27 is a multifunctional protein that regulates gene expression at transcriptional and post-transcriptional levels (reviewed in 1). HSV expresses over 80 transcripts during lytic infection and only four of them undergo splicing, the remainder being intronless. ICP27 inhibits host cell splicing, shuttles from nucleus to the cytoplasm and binds intronless viral RNAs. However, ICP27 does not bind intron containing HSV transcripts which accumulate in nuclear clumps (2), suggesting that IC27 mediates nuclear export of intronless viral RNAs (3).

Nuclear export of ICP27 is blocked by the drug leptomycin B (LMB) indicating a requirement of the export receptor CRM-1 for nuclear exit. Intriguingly, treatment with LMB blocks the accumulation of certain intronless HSV transcripts in the cytoplasm, but not all of them (4), thus some intronless RNAs may not require ICP27 for their export and are using an alternative pathway. Since the export of many cellular RNAs appears to be directly dependent upon splicing, ICP27 provides an interesting tool to investigate alternative mammalian RNA export pathways.

Our recent data show that ICP27 interacts with a constitutive splicing factor, SAP145, that splicing is inhibited before the first step in catalysis and ICP27 is detected co-migrating with the splicing complexes formed. Also we have shown that ICP27 interacts with the ubiquitous casein kinase 2 (CK2), hnRNP K (5) and REF-2, an hnRNP-like protein. Early in HSV infection, CK2 activity is upregulated by activating the α'-subunit and CK2 holoenzyme redistributes from nucleus to the cytoplasm (probably altering its substrate preference); export of ICP27 is important for both these effects. In turn, activated CK2 phosphorylates ICP27 and affects its export as well as phosphorylating hnRNP K which is not ordinarily a CK2 substrate. HnRNP K contains a novel KNS shuttling sequence that mediates export via a CRM-1-independent pathway.

REF was recently shown to participate in mRNA nuclear export through interaction with TAP, probably facilitating the interaction of TAP with cellular mRNAs (5). Although TAP does not seem to mediate cellular mRNA export by binding to mRNA, it binds to a constitutive transport element (CTE) of the simian type D retroviruses and promotes CTE-dependent export from the nucleus (6). When RNA from the late Us11 HSV gene, which is responsive to LMB treatment, was injected into Xenopus oocytes in the presence or absence of injected recombinant ICP27 we found that ICP27 dramatically stimulated the export of

Us11 RNA without affecting export of cellular mRNA, U snRNA or tRNA. TAP protein further stimulated the export of viral RNA, while an excess of CTE RNA saturated its export.

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Mechanisms of alternative splicing regulation in *Drosophila* sex determination and programmed cell death

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We are interested in the molecular mechanisms that regulate alternative pre-mRNA splicing during cell differentiation and development. Our approach has been to apply biochemical methods to study genetically well characterized systems of splicing regulation that control sex determination and dosage compensation in *Drosophila*. The protein Sex-lethal (SXL) binds to U-rich sequences and induces female-specific patterns of alternative splicing on at least three target genes: *transformer* (*tra*), *male-specific-lethal-2* (*msl-2*), and its own gene (Figure 1).

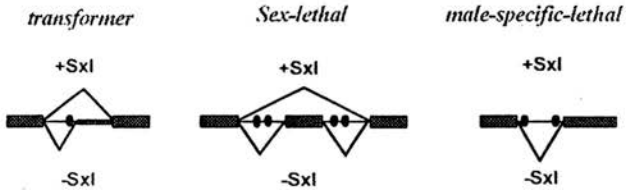

Figure 1: patterns of alternative pre-mRNA splicing induced by SXL in female *Drosophila* flies. Circles represent SXL binding sites.

In vitro and *in vivo* data are consistent with a model in which SXL binding to long, U-rich polypyrimidine(Py)-tracts associated with 3' splice sites (ss) of *tra* and *msl-2* prevents the association of the splicing factor U2AF, thereby inhibiting assembly of splicing complexes (1-3). U2AF is a heterodimer of 65 and a 35 kDa subunits. U2AF65 binds to Py-tracts and U2AF35 to the AG dinucleotide at the 3' end of the intron (4-6). In the case of *tra*, blockage of U2AF65 binding to the Py-tract of a non-sex-specific site is sufficient to divert U2AF to a downtream site of lower affinity, thus causing a switch in 3' ss utilization.

In contrast, retention of an intron in *msl-2* pre-mRNA demands a tighter control by SXL. First, an unusually long distance between the Py-tract and the 3' ss AG is required to prevent stabilization of U2AF65 binding to the Py-tract afforded by the interaction between U2AF35 and the 3' ss (3). Second, inhibition of U1 snRNP binding to the 5' ss by SXL is also required for efficient intron retention. To achieve this, SXL binds to a U-rich sequence immediately downstream of the 5' ss. Surprisingly, this sequence is also required in the absence of SXL for U1 snRNP binding to *msl-2* 5' ss. *In vitro* analyses using HeLa nuclear extracts revealed that the RNA binding protein TIA-1 binds to this U-rich stretch and promotes U1 snRNP recruitment. TIA-1 function is only required for U1 snRNP binding to weak 5' ss followed by U-rich stretches.

TIA-1 was identified previously as a factor that promotes apoptosis (7). Sequence comparisons revealed striking similarity between the 5' ss of *msl-2* and a 5' ss present in the pre-mRNA of the Fas receptor, which is alternatively spliced to generate mRNAs encoding either a membrane-bound apoptotic receptor or a soluble form that prevents apoptosis. TIA-1

influences this alternative splicing event by preventing accumulation of the mRNA encoding the apoptosis inhibitor, thus offering an explanation for the activity of TIA-1 in programmed cell death. In contrast to *msl-2*, where TIA-1 is required for splicing, TIA-1 modulates Fas splicing by influencing which 3' ss partner is chosen by the regulated 5' ss. Structural and functional similarities between TIA-1 and the *Saccharomyces cerevisiae* splicing factor Nam8 (8) suggest striking evolutionary conservation of a regulatory mechanism that can control biological processes as diverse as meiosis in yeast, dosage compensation in *Drosophila* or programmed cell death in humans.

The function of SXL in *tra* and *msl-2* regulation can be viewed as a steric block to the access of splicing factors to particular splice sites. This type of mechanism, however, cannot explain how SXL promotes an exon skipping event on its own pre-mRNA, because the binding sites for SXL are at considerable distances from the regulated splice sites. An amino-terminal glycine- and asparragine-rich (GN) domain is necessary and sufficient for SXL autoregulation. Our data suggest that the GN domain interferes with molecular events occurring in the transition between initial splice site communication across the exon and splice site pairing leading to intron removal.

Genetic data have implicated the gene *female-lethal(2)d* in *Sxl* autoregulation (9). Cloning and expression analyses indicated that FL(2)D is a nuclear protein (10) that forms a complex with SXL and other regulatory factors. Apart from SXL-regulated loci, FL(2)D associates with other nascent transcripts, opening the possibility that the protein acts as a cofactor for regulation of a variety of RNA processing events.

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Interconnections between polyadenylation, splicing and transcription in eukaryotic genes

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1) Messenger RNA 3' end processing and PolII transcription (1).
Our recent results show that transcriptional termination depends on both poly(A) signals, their associated RNA processing factors and downstream transcriptional pause sites, as judged by experiments performed in both yeast and higher eukaryotes (2-4).

In *S.pombe* we have set up a genetic screen to identify termination factors (5). We show that the C terminal domain (CTD) of the cleavage polyadenylation factor, CstF-64 (*RNA15* in yeast) has a termination function since its deletion uncouples mRNA 3' end processing (which works efficiently without it) from transcription termination. We have gone on to identify a second termination factor that interacts with this CTD called Res2. This protein is also part of a transcription factor called MBF specifying expression of genes regulated during the G1 to S phase of the cell cycle (6). We are currently studying how Res2 performs its second function in transcriptional termination.

In higher eukaryotes we continue to investigate PolII termination using both *in vivo* and *in vitro* systems. We have obtained new information on the molecular basis of human β-globin gene termination (4), as well as continuing to exploit our *in vitro* co-transcriptional polyadenylation and termination system (7,8). We hope that a combination of yeast genetics and coupled *in vitro* processing /transcription systems will enable us to uncover the molecular details of this basic gene expression mechanism.

2) Mechanism of poly(A) site regulation in retroviruses.
In the HIV-1 proviral genome, the poly(A) site in the 5' long terminal repeat (LTR) is inactivated by the proximity of U1SnRNP recruited to a strong splice donor site positioned 200nt downstream (9,10). This same poly(A) signal is by necessity highly active in the 3' LTR. We have investigated poly(A) site regulation in a second retrovirus, Moloney leukaemia virus (MoMLV). Even though the same arrangement of poly(A) site and donor site exists in MoMLV as in HIV-1, the MoMLV poly(A) site works efficiently in the 5' LTR resulting in a large fraction of MoMLV transcripts that fail to read through the 5' LTR region. We are currently investigating the molecular basis for the difference in 5' LTR poly(A) site regulation between HIV-1 and MoMLV. This inefficient approach to mRNA processing adopted by MoMLV must severely restrict its level of expression, a fact of relevance to the use of this virus as a gene therapy vector.

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Session 5
Chair: Jean-Dominique Vassalli

The function and intracellular localization of proteins that function early during spliceosome assembly

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During the first step of spliceosome assembly U1 snRNP recognizes the 5' splice site, whereas human (Hs) SF1 and U2AF^{65} bind to the branch site and the polypyrimidine tract upstream of the 3' splice site, respectively. SR proteins facilitate the binding of U1 snRNP to the pre-mRNA and bridge the splice sites by simultaneous interactions with the U1-specific 70K protein and U2AF^{35}. Structural and functional domains in SF1 are highly conserved from yeast to man (1, 2). They include an N-terminal domain that interacts with the third RNA recognition motif (RRM3) of U2AF^{65}, a hnRNP K homology (KH) domain that contacts the branch site, and one or two zinc knuckles that increase the affinity of SF1 for the branch site. These domains are sufficient for function of SF1 in spliceosome assembly *in vitro*, whereas the C-terminal Pro-rich half is dispensable for activity (2).

Drosophila (Dm) and *Caenorhabditis* (Ce) SF1 contain an additional N-terminal region enriched in Ser, Arg, Lys and Asp residues (3). This domain is similar to the RS domains present in SR and related proteins, which mediate protein-protein or protein-RNA interactions. The RS domains of DmSF1 and U2AF^{65} contact each other, which interferes with the interaction between DmSF1 and RRM3 of U2AF^{65}. Thus, the SF1-U2AF^{65} interaction, which is essential for spliceosome assembly in humans, may be modulated by the presence of the SF1 RS domain in flies. Moreover, DmSF1 and CeSF1 interact with the RS domain-containing proteins ASF/SF2 and U1-70K. Phosphorylation of the RS domain of DmSF1 in a *Drosophila* S100 extract or by SRPK1 enhances binding of DmSF1 to ASF/SF2, but reduces the interaction with U1-70K and has no effect on the DmSF1-DmU2AF^{50} interaction. Given that HsSF1 does not interact with ASF/SF2 or U1-70K, these data reveal a novel function for SF1 in flies, which may be subject to regulation. These data suggest that interactions which occur early during spliceosome assembly in humans are (at least in part) different in *Drosophila*.

Complex E is converted into pre-spliceosomal complex A by binding of the U2 snRNP to the branch site. The active 17S U2 snRNP is associated with two essential splicing factors, SF3a and SF3b (4 ,5). Analysis of functional domains in the three SF3a subunits has revealed that SF3a60 and SF3a66 interact with SF3a120 through their N-terminal portions but do not contact each other directly (6). Furthermore, all SF3a subunits bind individually to the 15S U2 snRNP/SF3b complex, albeit with different efficiencies. Zinc fingers present in SF3a60 and SF3a66 are essential for the integration into the 17S U2 snRNP and proper interactions between all subunits are necessary for full reconstitution of the active U2 snRNP. As expected, pre-spliceosome formation *in vitro* requires all SF3a subunits and, in particular, the zinc finger regions and the SF3a120 interaction sites of SF3a60 and SF3a66.

To study the assembly of the active U2 snRNP, we have analyzed the intracellular localization of the SF3a subunits by immunofluorescence and in living cells (7). Immunolocalization studies indicate that all subunits, similar to other splicing components,

are present in nuclear speckles and also show a diffuse distribution throughout the nucleoplasm. In contrast to U2 snRNA, Sm proteins and U2 B", the SF3a subunits are absent from Cajal bodies, suggesting that the U2 snRNP present in these structures represents an inactive form of the particle. Transiently expressed SF3a subunits fused to the green fluorescent protein colocalize with the endogenous counterparts and sometimes also accumulate in Cajal bodies. Transient expression of truncated versions of SF3a60 and SF3a66 indicated that the N-terminal domains required for interaction with SF3a120 are essential for nuclear targeting, suggesting that the formation of the SF3a complex is a prerequisite for the import of the particle into the nucleus. Deletion of the zinc fingers of both SF3a60 and SF3a66 results in an accumulation in the Cajal bodies. Together with the result that the zinc fingers are essential for the integration of SF3a60 and SF3a66 into the mature U2 snRNP, this observation supports a model, where the final steps of U2 snRNP assembly occur in the Cajal body.

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Synthesis and degradation of poly(A) tails

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The 3'-ends of almost all eukaryotic mRNAs are generated in a two-step reaction, endonucleolytic cleavage followed by the addition of a poly(A) tail. In mammalian cells, addition of a poly(A) tail requires three proteins, poly(A) polymerase, cleavage and polyadenylation specificity factor (CPSF) and poly(A) binding protein II (PABP 2). CPSF binds the polyadenylation signal AAUAAA, and PABP 2 binds the growing poly(A) tail. Together, the two RNA binding proteins tether the poly(A) polymerase to the RNA, leading to processive poly(A) synthesis. Processive synthesis stops after about 250 to 300 adenylate residues have been added to the pre-mRNA. This corresponds to the length of a newly synthesized poly(A) tail *in vivo*.

On high molecular weight poly(A), PABP 2 forms regular particles of a defined diameter of 21 nm. The length of poly(A) required for the formation of one full-sized particle corresponds to the length polymerized in the processive reaction, suggesting that particle formation may be involved in length control.

We are carrying out equilibrium studies on the PABP 2 - poly(A) interaction by fluorescence techniques to learn more about particle formation.

By mutagenesis, we have defined a region in PABP 2, likely an amphipathic alpha-helix, that is necessary but not sufficient for the stimulation of poly(A) polymerase, probably by direct interaction.

Two domains of PABP 2 contribute to RNA binding. One is an RNP domain, the second is arginine-rich. The latter contains thirteen asymmetrically dimethylated arginine residues, a modification considered a hallmark of RGG domains. However, the methylated arginines in PABP 2 are not in RGG sequences.

Poly(A) tails of a defined length are important for the cell since the rate of deadenylation is the first and often rate-limiting step of mRNA decay. We have described an enzyme, the poly(A)-specific ribonuclease, that catalyzes this reaction in Xenopus oocytes and possibly in mammalian somatic cells. Surprisingly, this 3'-exonuclease is stimulated by a 5' cap.

The role of SR proteins in the regulation of alternative splicing

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The SR proteins constitute a large family of nuclear phosphoproteins which are required for constitutive splicing and also influence alternative splicing regulation. They are highly conserved among species, with individual members of this family of proteins showing higher homology across species than to other proteins within the same family (1,2).

It was initially suggested that SR proteins were functionally redundant in constitutive splicing. However, differences have been observed in splicing commitments assays and in alternative splicing regulation, suggesting unique functions for individual SR proteins. The notion that SR proteins have unique functions is further supported by findings that *SF2/ASF*, *SRp20* and *B52*, the *D. melanogaster* homologue of *SRp55*, are essential genes. We decided to use the nematode *C. elegans* as a model organism to functionally characterize the SR family of proteins, and to address the important issue of functional redundancy. An advantage of the *C. elegans* system is the possibility of selectively inhibiting gene expression by RNA interference (RNAi). Homology searches of the *C. elegans* genome identified seven genes encoding putative orthologues of the human factors *SF2/ASF*, *SRp20*, *SC35*, *SRp40*, *SRp75*, and *p54*, and also several SR-related genes, including the homologues of *tra-2β* and *SRPK*, an SR protein kinase. Transgenic worms expressing a GFP reporter fused to SR promoters were used to analyze CeSR gene expression. RNAi of *CeSF2/ASF* caused late embryonic lethality, suggesting that this gene has an essential function during *C. elegans* development. In contrast, when the expression of every other nematode SR protein was individually suppressed by RNAi, no phenotypes were observed (3). This strongly suggests functional redundancy among the different members of this family of proteins. Simultaneous interference of two or more SR proteins in certain combinations caused lethality or other developmental defects. In summary, we show that CeSF2/ASF has unique properties and is essential for viability, and that functional redundancy exists for other nematode SR proteins.

Individual members of the SR and hnRNP A/B families of proteins have antagonistic roles in the regulation of pre-mRNA alternative splicing. The relative abundance of each SR protein and the molar ratio of each SR protein to hnRNP A1, or to other antagonists, has been proposed as a general mechanism for tissue-specific or developmental regulation of alternative splicing (4).

We have discovered an novel mechanism to regulate the levels of antagonistic splicing factors in the nucleus. We have found that exposure of different cell lines (3T3, COS, 293) to stress stimuli (osmotic shock, UVC-irradiation), but not to mitogenic activators such as PDGF or EGF, results in a marked cytoplasmic accumulation of hnRNP A1, concomitant with an increase in its phosphorylation. hnRNP B1 which shares a high degree of sequence similarity with hnRNP A1 also relocalizes to the cytoplasm, whereas SR proteins and other hnRNP proteins do not significantly alter their cellular distribution upon stress stimuli. The stress-induced change in the subcellular distribution of hnRNP A1 is mediated by phosphorylation of hnRNP A1 through the classical stress-response pathway involving $MKK_{3/6}$ and p38, and correlates with changes in alternative splicing of cotransfected reporters. This represents a

novel physiological mechanism, by which a signal transduction pathway alters the nuclear ratios of antagonistic splicing factors and modulates alternative splice site selection *in vivo* (5).

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Auto-regulation of ADAR2 alternative splicing by RNA editing

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ADAR2 is a double-stranded RNA-specific adenosine deaminase involved in the editing of mammalian mRNAs by the site-specific conversion of adenosine to inosine. RT-PCR amplification of rat brain RNA has identified four cDNAs encoding rat ADAR2 (rADAR2) isoforms produced as a result of two distinct alternative splicing events. One such splicing event uses of a more proximal 3'-acceptor site, adding 47 nucleotides (nt) to the mature rADAR2 mRNA. Nucleotide sequence analyses of rADAR2 genomic DNA have revealed the presence of AA and AG dinucleotides at the proximal and distal alternative 3'-acceptor sites, respectively. However, sequence comparisons between genomic DNA and cDNAs generated from rADAR2 primary RNA transcripts have indicated an A-to-G discrepancy for the more proximal 3'-splice site. Use of this 3'-acceptor is dependent upon the ability of rADAR2 to edit its own pre-mRNA, converting an intronic AA dinucleotide to an AI which can effectively mimic the conserved AG sequence normally found at 3' -splice sites.

Splicing to the proximal acceptor alters the reading frame of the mRNA and is predicted to generate a small inactive peptide rather than the catalytically-active 78 kDa protein produced in the absence of such an insertion. We hypothesize that the editing of rADAR2 primary RNA transcripts represents a negative autoregulatory mechanism by which editing and alternative splicing can decrease rADAR2 protein levels, thereby representing a novel mechanism by which this deaminase may limit its own expression and prevent excessive editing at sites that normally are modified by this deaminase or prevent editing of aberrant sites that are not modified *in vivo*.

We have taken advantage of an *in vitro* editing system using purified, recombinant ADAR2 protein expressed in *Pichia pastoris* and a rADAR2 minigene transcript extending from exon 4 through exon 5 (the region containing the alternative 3'-acceptor sites) to examine whether increased levels of ADAR2 will result in aberrant editing at sites not normally modified in rADAR2 pre-mRNAs isolated from rat brain. *In vitro* reaction products were amplified by RT-PCR, subcloned into a standard prokaryotic cloning vector and individual cDNA isolates were subjected to DNA sequence analysis. Results from these studies demonstrated a dose-dependent response in which low levels of recombinant rADAR2 were capable of modifying only a subset of those sites normally observed *in vivo*, whereas increased levels of rADAR2 resulted in A-to-I modifications for novel adenosine moieties within the RNA substrate.

To examine the consequences of rADAR2 overexpression in an animal model system, we have generated transgenic mice that express epitope (FLAG)-tagged rADAR2 under the control of human cytomegalovirus promoter. Founder mice were screened for the presence of the transgene by Southern blotting analysis. Four of twenty-eight founder mice carried the transgene and demonstrated varying levels of obesity. Initial feeding studies suggested that this obesity was a result of hyperphagia, as well as yet unidentified metabolic defect(s) that produced an excessively fatty liver during early postnatal development. These studies indicate that the ability of ADAR2 to limit its own expression may represent a critical regulatory mechanism that prevents the generation of aberrantly modified RNA species that can have deleterious consequences for an organism.

Nuclear RNA turnover

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We have previously reported the identification of the exosome complex (Allmang *et al.*, 1999b; Mitchell *et al.*, 1997). Ten components of this complex are predicted or shown to have 3'→5' exonuclease activity, and eight of these are homologous to characterized 3'→5' exonucleases from *E.coli*. Nuclear and cytoplasmic forms of the complex exist, each with eleven components, that differ by the presence of Rrp6p in the nuclear complex and Ski7p in the cytoplasmic complex. The nuclear exosome functions in the synthesis of ribosomal RNA (rRNA), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs), and degradation of pre-rRNA spacer regions (Allmang *et al.*, 1999a; Allmang *et al.*, 2000; Briggs *et al.*, 1998; van Hoof *et al.*, 2000), while the cytoplasmic exosome participates in mRNA turnover (Anderson and Parker, 1998).

We have now shown that the nuclear exosome also degrades nuclear pre-mRNAs (Bousquet-Antonelli et al. 2000). Inhibition of pre-mRNA degradation resulted in increased levels of both unspliced pre-mRNAs and spliced mRNAs, indicating that pre-mRNA degradation is a normal feature of gene expression. When splicing was inhibited by mutation of a splicing factor, inhibition of turnover resulted in 20 to 50 fold accumulation of pre-mRNAs, accompanied by increased mRNA production. Splicing of a reporter construct with a 3' splice site mutation was also increased on inhibition of turnover, showing competition between degradation and splicing. Nuclear RNA turnover also involves 5'→3' degradation by the exonuclease Rat1p. Like the exosome, Rat1p participates in pre-rRNA and pre-snoRNA processing and pre-rRNA spacer degradation (Allmang *et al.*, 1999a; Allmang *et al.*, 2000; Henry *et al.*, 1994; Petfalski *et al.*, 1998; Villa *et al.*, 1998). Rat1p is substantially homologous to Xrn1p (Kenna *et al.*, 1993), which plays a major role in cytoplasmic mRNA turnover. Analysis of pre-mRNAs in which progression of the exonucleases was inhibited by the presence of an intron-encoded snoRNA, indicated that 3'→5' degradation by the exosome is normally the major pathway. This is in contrast to cytoplasmic mRNA turnover in which 5'→3' degradation predominates, at least in yeast.

Nuclear 3'→5' degradation is regulated in response to carbon source. Degradation activity was low on carbon sources that are converted to glucose within the cell: galactose, maltose or acetate, and was strongly activated on media containing glucose, or sugars that are converted to glucose by extracellular enzymes: raffinose or sucrose. This indicates that the availability of extracellular glucose up-regulates 3'→5' nuclear pre-mRNA degradation. Many other metabolic activities are regulated by extracellular glucose in yeast, via two well characterized signal transduction pathways. Whether these directly modify exosome components remains to be determined. This regulation appears to be specific, since other "housekeeping" activities of the exosome, in rRNA, snRNA and snoRNA synthesis, are not co-regulated. We propose that nuclear pre-mRNA turnover represents a novel step in the regulation of gene expression

Human homologues have been identified for most exosome components and four of these have been shown to function in yeast. All tested human components are present in a large complex that is recognized by PM-Scl autoimmune antibodies (Briggs *et al.*, 1998; Allmang *et al.*, 1999). This complex is very likely to be the human exosome. As in yeast, nuclear and cytoplasmic forms of the human exosome exist; only the nuclear complex contains PM-Scl100 , the homologue of yeast Rrp6p.

Previous turnover data indicated that the degradation of nuclear pre-mRNA (or hnRNA) is very active in human cells. Labeling data showed that only around 2% of the hnRNA population was converted to cytoplasmic mRNA. Both Rat1p and exosome components are functionally conserved from yeast to humans, indicating that the nuclear RNA turnover pathway is likely to be conserved. Moreover, alterations in the balance between pre-mRNA processing and degradation is likely to have greater consequences in humans and other metazoans, where regulated and alternative splicing is a common and important feature of gene expression.

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POSTERS

RNA editing and splicing of glutamate receptor pre-mRNA

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RNA editing by adenosine deaminase that act on RNA (ADAR) requires RNA that is in an extensively double-stranded conformation to perform its action of converting adenosines to inosines. There are at least two ADAR enzymes that possess this activity, ADAR1 and ADAR2. Depending on the extent of the double-stranded structure of the substrate, promiscuous or selective editing occur. Promiscuous editing occurs on almost completely base paired RNA substrates such as RNA viruses, where up to 50% of the adenosines can be edited. Selective editing has mainly been found in nuclear-encoded RNA involved in neurotransmission. In these substrates double-stranded regions are interrupted by bulges or loops and only a few specific sites are edited. We are focusing on the mechanism of selective editing of the glutamate receptor subunit B, GluR-B, in rat. The GluR-B pre-mRNA is edited at two sites that give rise to an amino acid change, the Q/R and R/G site, referring to the codon alteration. To understand the mechanism of selective editing we have chosen to focus on editing of the R/G site situated in the stem of a 68 nucleotides long stem loop. Our results show that ADAR2 can edit the R/G site selectively in vitro while ADAR1 edits more promiscuously at several other sites. ADAR2 editing is also consistent with in vitro editing using HeLa nuclear extract and endogenously edited RNA. This indicates that ADAR2, opposed to ADAR1, can selectively edit a natural substrate in vitro without additional factors. Further, we have observed that it is the sequence rather than the presence of bulges in the stem that determines the specificity since a mutant lacking the bulges still is edited selectively by ADAR2. We are also interested in the relation between editing, splicing and the transcription machinery. In order to achieve editing at the R/G site an editing complementary sequence located in the downstream intron is required. In general intron sequences are required for editing to occur on endogeniously encoded RNA and it is therefore necessary that editing happen before splicing. The intron located one nucleotide downstream of the R/G site is spliced very poorly in vitro using the natural 3' splice site. One explanation for this is a possible sequestering of the 5' splice site in the stable R/G stem-loop structure. The stem-loop is also phylogenetically conserved. However, we have found that splicing inhibition can be overcome by exchanging the 3' splice site with a more efficient one. We are currently investigating if a more efficiently spliced intron has an effect on editing in order to characterize the relationship between ADAR editing and splicing.

Control of HPV-16 L1 production caused by an RNA instability element

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The HPV life cycle is strictly linked to the differentiation state of the cell. In the case of HPV-16 only small quantities of virus is produced in vivo. We have previously shown that both L1 and L2 coding regions contain intragenic negative elements which reduce the production of capsid protein (1), (2). In this study we show that when L1 is transfected in tandem with Equine Infectious Anaemia Virus (EIAV) gag expression plasmid, an mRNA which contains no negative elements, EIAV mRNA is expressed in large quantities whereas L1 mRNA is undetectable. This effect is seen in both cytoplasmic and total RNA fractions. In an attempt to map this element we constructed L1-EIAV hybrids and analysed the mRNA stabilisation through northern blots. Results obtained from hybrid transfections and northern blot analysis showed that the 5'-end of the HPV-16 L1 coding sequence contains an element with strong destabilising functionality. In contrast, the 3'-end of the L1 sequence did not appear to alter mRNA levels. Further work mapped the boundaries of the negative element and site specific mutagenesis allowed fine mapping of its structure. We have also identified proteins which interact specifically with the minimal negative element. HPV-16 has most likely evolved in such a manner as to utilise its negative element to an advantage over the host it infects.

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Identification of the mammalian homologue of the splicing regulator suppressor-of-white-apricot as a thyroid hormone regulated gene

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Thyroid hormone (T3) is an important regulator of mammalian brain maturation. Lack of adequate levels of T3 during the fetal and neonatal periods leads to mental deficiency in humans and multiple severe abnormalities. In order of understand better these alterations and the effects of T3 in he brain we have searched for new T3-regulated genes in this organ. For this purpose we have used the differential display PCR method in the T3 responsive neuronal cell line E18 + TRa. We have identified the rat homolog of the Drosophila splicing regulator SWAP (Suppressor of white apricot) as a thyroid hormone regulated gene in the developing rat brain and in the E18 + TRa cells. We found that hypothyroidism causes an abnormal high level of SWAP RNA between postnatal days 5 and 15 throughout the brain except the cerebellum. A subset of T3-regulated genes contains no T3 response elements, suggesting a role of this hormone in post-transcriptional regulation mechanisms. In base of our results with SWAP we have studied the effect of thyroid status on the expression of several genes involved in this kind of processes (mRNA stability and RNA splicing), as well as the mechanism by which these genes modulate others that are under thyroid control during the brain development. By modulating the expression of SWAP and other mRNA stability and splicing regulators thyroid hormone may exert wide regulatory effects on multiple genes. The regulation of SWAP gene defines a novel mechanism of action of thyroid hormone which can be important for its effects in the developing brain.

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A mammalian germ cell-specific RNA binding protein interacts with ubiquitously expressed proteins involved in splice site selection

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At least 22% of human genes are alternatively spliced, which makes the genome sequence the tip of the iceberg in terms of informational complexity. Very little is known about how these splicing pathways are selected both within and between different cell types. One good candidate regulatory protein for controlling germ cell-specific splicing is RBMY. Within human germ cells RBMY protein shows a similar nuclear distribution to components of the pre-mRNA splicing machinery. We have used protein-protein interaction assays to test for possible physical interactions between these proteins. We find that RBMY protein directly interacts with members of the SR family of splicing factors, and in addition strongly interacts with itself. We have mapped the protein domains responsible for mediating these interactions, and expressed the mouse RBMY interaction region as a bacterial fusion protein. This fusion protein can pull down several functionally active SR protein species from cell extracts. Depletion and add-back experiments indicate that these SR proteins are the only splicing factors bound by RBM which are required for the splicing of a panel of pre-mRNAs. Our results suggest that RBMY protein is an evolutionarily conserved mammalian splicing regulator which operates as a germ-cell specific co-factor for more ubiquitously expressed pre-mRNA splicing activators. These studies may indicate how splicing pathways operate in other cell types. *RBMY* is closely related to the X chromosome gene encoding heterogeneous ribonucleoprotein (hnRNP) G and a new member of this gene family, *HNRNP G-T* which we have mapped it as a single copy gene on chromosome 11. As a family these proteins interact with pre-mRNA splicing factors and signal transduction proteins. Differentially expressed hnRNPG-like proteins might modulate different splicing pathways in different tissues, and in response to different signal transduction events. We will also present evidence that new cell type-specific functional copies of this gene family have been generated as a result of retrotransposition events.

Alternative splicing of c-H-ras a system to study different aspects of the splicing mechanism.

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We studied the alternative splicing of the c-H-ras gene that can include or not the alternative exon IDX. The inclusion of IDX renders a protein with low tumorigenic activity. *In vitro* splicing assays in HeLa nuclear extracts with mini genes showed that the ras exon 4 ligates preferentially with ras exon 3 (distal) instead of ras IDX (proximal). We mutated exon 3 to a very small non-related exon of 11 nucleotides (nt), which was 5' terminal. Surprisingly, exon 4 ligated with both the 11 nt artificial exon and the proximal IDX exon. The splicing of the 11 nt that rendered 11nt-exon 4 depended on the Cap Binding Complex (CBC), and furthermore it was inhibited with ApppG and m_3GpppG capped pre-mRNAs, while the splicing that includes the IDX proximal exon was not. Mutation of the 5' splice site of the 11 nt exon (distal) did not increase the rate of inclusion of the IDX exon. In conclusion: 1) CBC complex only regulates the inclusion of the 11 nt exon but not that of the proximal exon; 2) The splicing that includes the IDX exon is repressed in HeLa nuclear extracts independently of competing 5' distal exons.

Herpes simplex virus protein ICP27 inhibits pre-mRNA splicing *in vitro*

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We use Herpes simplex virus (HSV) as a model system to study the mammalian pre-mRNA splicing machinery. During a productive HSV infection splicing of host cell pre-mRNAs are impaired. Considerable amount of data suggests that the immediate early HSV protein ICP27, directly or indirectly, is responsible for the shut down of splicing. We have developed an in vitro splicing assay in order to test whether ICP27 in the absence of other HSV proteins is inhibitory for the splicing process and to identify components of the splicing machinery that are targets for ICP27. The in vitro splicing assay relies on nuclear extracts prepared from HeLa cells in which we have transiently expressed ICP27 from a recombinant adenovirus vector. Using this assay we find that splicing activity is lower in ICP27 containing nuclear extracts than in control extracts. RNA polymerase II activity, on the other hand in, are comparable in ICP27-extracts and control extracts, suggesting a specific effect on the splicing process rather than a toxic effect from an over-expressed protein. Thus, ICP27 on its own, in the absence of other HSV proteins may cause splicing inhibition. Moreover, we find that the observed splicing inhibition in ICP27-extracts is reversed by a small addition of control extract, indicating that it is a limiting component of the splicing apparatus that is targeted by ICP27. We are currently analysing at which stage of the splicing process the ICP27 caused inhibition is first manifested as well as trying to identify splicing components that are targeted ICP27.

WT1: a transcription factor with a splicing connection?

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The tumour suppressor gene *WT*1 has been implicated, by mutational analysis, in 10-15% of Wilm's tumours, a pediatric malignancy which affects 1 in 10,000 children. *WT*1 has a key role in urogenital development, and is expressed in a number of tissues of mesodermal origin which undergo a mesenchyme to epithelium transition. The WT1 protein contains four C-terminal (Cys_2-His_2) zinc-fingers of the "Krüppel" type, closely related to those present in the transcription factor EGR1. In the N-terminal region, WT1 possesses a Q/P-rich trans-regulatory domain and a putative RRM. Isoforms arise from alternative splicing events: of particular interest is exon 9, which encodes only three amino-acids (KTS). Intriguingly, recent findings suggest that whereas WT1 (-KTS) acts as a transcription factor, WT1 (+KTS) is involved in splicing. WT1 is now part of an increasing list of multifunctional proteins which, in all likelihood, connect the different steps of gene expression.

In order to investigate the role of WT1 in this context, we have used a variety of biochemical fractionation techniques. Oligo(dT) and anion exchange chromatography, combined with density gradients, suggest that a substantial proportion of WT1 is present in RNase-sensitive nuclear poly(A)$^+$ RNP. In addition, the first two zinc fingers of WT1 are required for both nuclear import and incorporation into pre-mRNP. In the future, by combining these fractionation techniques with immunoprecipitation assays, we hope to identify target pre-mRNAs in physiologically relevant tissues. Finally, expression of T7-tagged isoforms in *Xenopus* oocytes provides supporting evidence for WT1 having a role in pre-mRNA biogenesis.

A switch in 3' splice site recognition during exon definition and catalysis is important for Sex-lethal autoregulation

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Maintenance of female sexual identity in Drosophila involves an autoregulatory loop in which the protein Sex-lethal (SXL) promotes skipping of exon 3 from its own pre-mRNA. Using transient transfection assays in Drosophila Schneider cells we have found that exon 3 repression requires competition between exon 2 and exon 3 5' splice sites, but is independent of their relative strength. Two 3' splice site AGs precede exon 3. We report here that while the distal site plays a critical role in defining the exon, the proximal site is preferentially used for the actual splicing reaction, arguing for a switch in 3' splice site recognition between exon definition and splicing catalysis. Surprisingly, the presence of the two 3' splice sites is important for efficient regulation by SXL, suggesting that SXL interferes with molecular events occurring between initial splice site communication across the exon and splice site pairing leading to intron removal.

The role of tri-snRNP proteins in [U4/U6.U5] tri-snRNP formation and integration into the spliceosome

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We have developed a method to dissociate HeLa 25S [U4/U6.U5] tri-snRNPs into stable 13S U4/U6 snRNPs and 20S U5 snRNPs. The purified U4/U6 and U5 snRNPs can be used to reconstitute a tri-snRNP particle in vitro in the absence of additional factors. Very little is known about the interactions that mediate the association of these two snRNP particles in human tri-snRNPs. We have characterised the U5-specific 102 kD protein, an ortholog of the S. cerevisae Prp6 protein, and shown that U5-102kD interacts within the tri-snRNPs with both the U5 and U4/U6 snRNPs, thus bridging the two particles. We have identified the protein composition of the 13S U4/U6 snRNP. The particle contains, in addition to the Sm proteins, a set of seven Sm-like proteins, and five specific proteins with molecular masses of 15.5, 20, 60, 61, and 90 kD. The 15.5 kD protein binds directly to the 5' stem-loop of U4 snRNA and the 20/60/90 kD proteins are snuCyp20, hPrp4p and hPrp3p, respectively. The novel 61 kD protein was identified as an ortholog of the yeast Prp31p. The 13S U4/U6 snRNPs were examined by electron microscopy, and the images obtained exhibit a bicycle-like structure, in which the two globular domains are likely to represent the Sm core and LSm complex. Based on biochemical data and electron microscopy studies, we propose a structural model for the 13S U4/U6 particle. Employing our tri-snRNP reconstitution assay we could demonstrate that at least three tri-snRNP-associated proteins with molecular masses of 65, 100 (human Prp28) and 110 kD are not required for tri-snRNP formation. We have characterised the 65 kD and 110 kD proteins in more detail and shown that both proteins are essential for pre-mRNA splicing in vitro and that they promote the stable integration of tri-snRNPs into the spliceosome.

Analysis of the effects of overexpression of the yeast poly(A)-binding protein, Pab1p

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In yeast, the addition of the mRNA poly(A) tail is a multi-step process with its final length determined by several factors, including Pab1p (1), its associated factor Pbp1p (2), and poly(A)-nuclease Pan2p/Pan3p (3). Expression of Pab1p is autoregulated, with the protein binding to an adenylate-rich region in the 5'-UTR of its mRNA. Over-expression of PAB1 produces only a very slight increase in Pab1p expression with no effect on cell growth. In contrast, expression of a PAB1 allele lacking its adenylate-rich region from the inducible GAL10 promoter, results in increased Pab1p levels and a slow growth phenotype. However, much of the overexpressed protein is proteolyzed. Interestingly, the amount of Pbp1p in these cells decreases, suggesting that proteins associated with Pab1p are degraded. Consistent with this observation, extracts from cells overexpressing Pab1p exhibit decreased polyadenylation to an extent comparable to that seen with a pbp1* extract. These results suggest that the slow growth phenotype is most likely due to a defect in mRNA processing. To more fully understand the action of Pab1p overexpression, we isolated high-copy suppressors of the slow growth phenotype. Six genes were identified which enabled rapid growth of the cells while maintaining high levels of Pab1p. Of these, RRP6 (4) and SPB4 (5,6) affect RNA processing and deletion of either gene suppresses a deletion of PAB1. Five of the genes identified also suppress the slow growth phenotype of a strain overexpressing PBP1, suggesting that overexpression of PAB1 and PBP1 affect the same cellular pathway(s).

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A human sequence homologue of Staufen is an RNA binding protein that is associated to polysomes and localizes to the rough endoplasmic reticulum

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The Influenza virus non-structural protein NS1 is an RNA-binding protein that may be involved in several regulatory processes during viral infection. These include the modulation of pre-mRNA splicing, the retention of poly A-containing RNA in the nucleus and the stimulation of viral mRNA translation. In the course of a two-hybrid screen to detect candidate cellular proteins that may pertain to these biochemical effects, a human clone was identified capable of coding for a protein with high homology to the Staufen protein from D. melanogaster (dmStaufen). Staufen protein is the product of a maternal mRNA of D. melanogaster that is involved in the accumulation of bicoid and oskar mRNAs at the anterior pole of the embryo and the posterior pole of the oocyte, respectively. It contains dsRNA binding domains that associate with bicoid mRNA through a precise secondary structure in its 3 UTR. In view of this homology, human cDNAs were isolated from a l cDNA library. The encoded protein (hStaufen-like) contained 4 dsRNA binding domains with 55% similarity and 38% identity to those of the dmStaufen, including identity at all residues involved in RNA binding. A recombinant protein containing all dsRNA binding domains was expressed in *E. coli* as a His-tagged polypeptide. It showed dsRNA binding activity *in vitro*, with an apparent Kd of 10-9 M. Using a specific antibody, a main form of the hStaufen-like protein could be detected in human cells, with apparent molecular mass of 60-65 kDa. The intracellular localization of hStaufen-like protein was investigated by immunofluorescence, using a series of markers for the cell compartments. Co-localization was observed with rough endoplasmic reticulum, but not with endosomes, cytoskeleton or Golgi apparatus. Furthermore, sedimentation analyses indicated that hStaufen-like protein associates with polysomes. Analyses of extracts from HeLa cells indicated that hStaufen-like protein is present in the cell in association with polysome complexes. As a whole, these results suggest that the human homologue of dmStaufen protein may be related to the localization and/or the translation of cellular mRNA.

Ski7p: An activator of the exosome for mRNA decay?

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One general pathway for mRNA turnover in yeast involves loss of the poly(A) tail followed by 3'->5' degradation of the deadenylated mRNA. The 3' decay of the mRNA body requires the exosome, a multienzyme complex of 3'->5' exoribonucleases, and the Ski2p, Ski3p and Ski8p proteins. The mechanism by which the 3' decay pathway is triggered upon removal of the poly(A) tail, and how exactly the exosome functions together with these factors, is not clear. Purified preparations of the exosome complex exhibit 3'->5' exoribonuclease activity in vitro. Biochemical fractionation experiments demonstrate that the enzymatic activity of the exosome is attributable to a subfraction of the total complex and that this activity is itself heterogeneous. These observations suggest that exosome activity available for a particular RNA processing pathway is limited by the availability of specific cofactors. We are currently analysing the protein composition of the active exosome fractions. Among the exosome-associated proteins identified in this work is Ski7p. In contrast, Ski2p, Ski3p and Ski8p were not identified. Notably, Ski7p has been proposed to be a limiting factor in Ski protein function. Ski7p is homologous to the translation elongation factor EF-1a (EF-Tu) and the elongation release factor eRF3, and contains all consensus sequences of GTPase proteins. The current data suggest models where the Ski7p GTPase activates the cytoplasmic exosome for the 3'->5' decay pathway. The in vivo function of Ski7p, and in particular its GTPase activity, in the 3'->5' mRNA decay pathway are being addressed.

The assembly of five proteins to the Drosophila polo pre-mRNA requires a pyrimidine rich region upstream of its poly(A) signal

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PTB (polypyrimidine tract binding protein) was previously shown to activate cleavage of the human C2 complement gene pre-mRNA through binding to an upstream sequence element (USE) (Moreira et al., Genes & Dev. (1998) 12: 2522-2534). In order to study the role of PTB on mRNA 3'end formation in other organisms, we have cloned Drosophila melanogaster PTB cDNA. In situ hybridisation on polythene chromosomes have localised this gene in the 100F region of the 3R chromosome and a protein is recognised and immunoprecipitated by the polyclonal anti-hPTB1 antibody from Drosophila protein extracts. The Drosophila polo gene encodes for a kinase involved in the regulation of the cell cycle progression and has two poly (A) signals. 125 nt upstream the first poly (A) signal there is a 27 nt region very rich in pyrimidines (24 Py). This region was mutated and U.V. cross-linking assays were performed using wild type and mutant precursor RNAs with HeLa and Schneider cells nuclear extracts. The mutation disrupts the binding of 5 proteins to the pre-mRNA. The identification and functional characterisation of these proteins is being performed at the moment in order to understand its role in mRNA 3'end formation in Drosophila.

Molecular characterization of the Drosophila fl(2)d gene, a cofactor of alternative pre-mRNA splicing regulation by Sex-lethal

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The Drosophila gene female-lethal(2)d [fl(2)d] interacts genetically with the master regulatory gene for sex determination Sex-lethal (Sxl). Both genes are required for the activation of female-specific patterns of alternative splicing on transformer and Sex-lethal pre-mRNAs. We have used P element-mediated mutagenesis to identify the fl(2)d gene. The fl(2)d transcription unit generates two alternatively spliced mRNAs that can encode two protein isoforms differing at their amino-terminus. The larger isoform contains a domain rich in histidine and glutamine, but has no significant homology to proteins in databases. Several lines of evidence indicate that this protein is responsible for fl(2)d function. FL(2)D protein can be detected in extracts from Drosophila cell lines, embryos, larvae and adult animals, without apparent differences between sexes. Consistent with a possible function in post-transcriptional regulation, FL(2)D protein has nuclear localization and is enriched in nuclear extracts. FL(2)D protein is associated with transcriptionally active loci in polytene chromosomes. Biochemical analysis and mass-spectrometry revealed that FL(2)D forms complexes with Sex-lethal, VIRILIZER and the Drosophila homologue of Ran Binding protein 7. The functional implications of these associations are currently under investigation.

Functional analysis of the *Drosophila Poly(A) polymerase* gene

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Translational control of maternal mRNAs plays an essential role in early development of many species. Maternal mRNAs are stored as dormant mRNAs that are translationally silent and are activated during early developmental events where they participate in oogenesis and embryogenesis. One mechanism to modulate maternal mRNA translation is cytoplasmic elongation of their poly(A) tails; dormant mRNAs have short poly(A) tails and their translational activation follows poly(A) tail elongation. Cytoplasmic polyadenylation can be reconstituted *in vitro* with two factors involved in nuclear polyadenylation, CPSF (Cleavage and Polyadenylation Specificity Factor) which binds to the poly(A) signal AAUAAA, and a poly(A) polymerase (PAP). This regulation also requires at least a specific protein, CPEB (Cytoplasmic Polyadenylation Element Binding Protein) which binds to CPEs in mRNAs whose poly(A) tail is to be elongated. Cytoplasmic polyadenylation has been described in *Drosophila*. Production in early embryos of Bicoid and Toll proteins, two major determinants of embryonic patterning, depends on cytoplasmic polyadenylation of the corresponding mRNAs.

As a starting point to study cytoplasmic polyadenylation in *Drosophila*, we have cloned and initiated a genetic analysis of *Pap*, the gene encoding *Drosophila* PAP. The *Pap* gene produces several mRNAs by alternative splicing and alternative poly(A) site utilisation. In contrast to what occurs in vertebrates where several isoforms of PAP have been described, *Drosophila Pap* mRNAs appear to encode a single protein. *Drosophila* PAP shares 56% overall identity with bovine PAP. Homology resides mostly in the N-terminal three-quarters of the protein. The *Drosophila* protein has the same activity as its bovine homologue in reconstituted polyadenylation assays. Using an antibody raised against *Drosophila* PAP, we show that the protein accumulates mostly in nuclei throughout *Drosophila* development, and is synthesised in large amount at the onset of zygotic transcription. We have generated several *Pap* mutants by mobilisation of a P element in the 5'-UTR of the gene. Strong *Pap* mutants are lethal at late embryonic stage. These mutants show a strong genetic interaction with a *orb* mutant; the *Drosophila orb* gene encodes a homologue of CPEB. Genetic interaction between *Pap* and *orb* is consistent with a role of both genes in cytoplasmic polyadenylation. Overexpression of *Pap* in the female germline is extremely detrimental to early development as this overexpression leads to early embryonic lethality of the progeny from such females. These embryos that do not develop, show a higher Bicoid gradient than wildtype embryos. We are currently examining to what extend cytoplasmic polyadenylation is affected in this context.

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88 **Workshop on Structure and Mechanisms of Ion Channels.**
Organizers: J. Lerma, N. Unwin and R. MacKinnon.

89 **Workshop on Protein Folding.**
Organizers: A. R. Fersht, M. Rico and L. Serrano.

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