Viruses as a Model System for Studies of Eukaryotic mRNA Processing

BY

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Viruses depend on their hosts for the production and spread of new virus particles. For efficient virus replication, the viral genes have adapted the strategy of being recognized and processed by the cellular biosynthetic machineries. Viruses therefore provide an important tool to study the cellular machinery regulating gene expression. In this thesis, we have used two model DNA viruses; herpes simplex virus (HSV) and adenovirus, to study RNA processing at the level of pre-mRNA splicing in mammalian cells.

During a lytic infection, HSV causes an almost complete shut-off of host cell gene expression. Importantly, HSV infection causes inhibition of pre-mRNA splicing which is possibly advantageous to the virus, as only four HSV genes contain introns.

The HSV immediate early protein, ICP27, has been shown to modulate several post-transcriptional processes such as polyadenylation and pre-mRNA splicing. We have studied the role of ICP27 as an inhibitor of pre-mRNA splicing. We show that ICP27 inhibits pre-mRNA splicing in vitro in the absence of other HSV proteins. We further show that ICP27 inhibits splicing at the level of spliceosome assembly. Importantly, ICP27-induced inhibition of splicing can be reversed, either by the addition of purified SR proteins, which are essential splicing factors, or by the addition of an SR protein-specific kinase, SRPK1. We propose that SR proteins are prime candidates as mediators of the inhibitory effect of ICP27 on pre-mRNA splicing.

In order to learn more about how splicing is organized in the cell nucleus in vivo, we investigated how cellular splicing factors are recruited to sites of transcription and splicing in adenovirus-infected cells using confocal microscopy. Our results showed that the SR proteins, ASF/SF2 and SC35, are efficiently recruited to sites in the nucleus where adenovirus genes are transcribed and the resulting pre-mRNAs are processed. Our results demonstrate that only one of the two RNA recognition motifs (RRMs) present in the ASF/SF2 protein is required for its recruitment to active sites of splicing. The arginine/serine rich (RS) domain in ASF/SF2 is redundant for the translocation of the protein to active viral polymerase II genes in adenovirus-infected cells.
To my family
Main references

This thesis is based on the following articles, which will be referred to in the text by their roman numerals.

I. Anette Lindberg and Jan-Peter Kreivi. 2002. Splicing Inhibition at the Level of Splicersome Assembly in the Presence of Herpes Simplex Virus Protein ICP27. Virology 294, 189-198

II. Anette Lindberg and Jan-Peter Kreivi. 2003. Herpes Simplex Virus protein ICP27 induced inhibition of splicing is reversed by SR-proteins. Manuscript

III. Anette Lindberg, Margarida Gama-Carvahlo, Maria Carmo-Fonseca and Jan-Peter Kreivi. 2003. A single RNA recognition motif in SR proteins directs them to nuclear sites of adenovirus transcription. Manuscript

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### 1. Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>ASF/SF2</td>
<td>alternative splicing factor/splicing factor 2</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DBP</td>
<td>adenovirus DNA binding protein</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>hnRNPs</td>
<td>heterogenous nuclear ribonucleoprotein particles</td>
</tr>
<tr>
<td>hpi</td>
<td>hours post infection</td>
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<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
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<tr>
<td>ICP27</td>
<td>infected cell protein 27, a herpes simplex virus protein</td>
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<tr>
<td>IGC</td>
<td>interchromatin granule clusters</td>
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<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NE</td>
<td>nuclear extracts prepared from HeLa cells</td>
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<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>RNA pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA Recognition Motifs</td>
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<tr>
<td>RS-domain</td>
<td>arginine- and serine-rich domain in the SR family of splicing factors</td>
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<tr>
<td>snRNPs</td>
<td>small nuclear ribonucleoprotein particles</td>
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<tr>
<td>SRrp</td>
<td>SR-related protein</td>
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<tr>
<td>SRs</td>
<td>serine-arginine proteins, splicing factors</td>
</tr>
<tr>
<td>S100</td>
<td>cytoplasmic extract prepared from HeLa cells</td>
</tr>
<tr>
<td>U2AF</td>
<td>U2 snRNP Auxiliary Factor</td>
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2. Introduction

The genetic information of eukaryotic cells is stored as double stranded DNA (dsDNA). The DNA is transcribed into a precursor messenger RNA (pre-mRNA), which is processed and subsequently translated into proteins. Most newly synthesised pre-mRNA contains stretches of non-coding RNA (introns) that must be excised by splicing before the RNA can be translated into a protein. Although many of the splicing factors and components of the splicing machinery are well characterized today, there are still gaps in our knowledge about the complex process of splicing. By alternative splicing, one gene can express more than one mRNA thus generating functionally diverse isoforms. Since the vast majority of human genes contain introns and most pre-mRNAs undergo alternative splicing, disruption of the normal splicing pattern is a common cause of human disease (reviewed in (Faustino and Cooper 2003) (Blencowe 2000)).

Viruses have been used as model systems to study eukaryotic gene regulation for decades. When a virus infects a cell, viral mRNA production is reliant, to a varying degree, on the cellular transcription machinery. In addition, the viral mRNAs must be translated by the cellular protein-synthesising machinery. Since viral genes have adapted to be recognized and processed by the cellular machinery, they represent an important tool to study cellular gene expression and regulation.

In order to study mRNA processing in mammalian cells at the level of mRNA splicing, we have used two model DNA viruses; herpes simplex virus-1 (HSV-1) and adenovirus. During lytic HSV-1 and adenovirus infections, almost all host cell gene expression is turned off. Results from different studies indicate that in the case of HSV-1, a viral protein termed ICP27 (Infected Cell Protein 27) blocks cellular gene expression by inhibiting pre-mRNA splicing (Sandri-Goldin and Mendoza 1992; Hardy and Sandri-Goldin 1994; Bryant, Wadd et al. 2001; Sciabica, Dai et al. 2003). As only four of approximately 80 HSV genes contain introns, the virus is relatively resistant to the inhibition of splicing. Projects I & II were initiated to investigate the role of ICP27 in the inhibition of pre-mRNA splicing. Our aims were to establish an in vitro splicing system that would allow for the characterization of how ICP27 inhibits the splicing process and reveal which cellular splicing factor(s) are targeted.

In project III, we used adenovirus as a model system to learn more about how splicing factors are organized in the cell nucleus in vivo. Relatively little is known about how splicing
factors are recruited to sites of active RNA polymerase II transcription and pre-mRNA splicing. Generally, splicing factors are found throughout the nucleoplasm, but many of them are concentrated in speckles. Numerous studies have concluded that inactive splicing factors are recycled and temporarily stored in speckles (Huang and Spector 1996; Gama-Carvalho, Krauss et al. 1997; Misteli and Spector 1998). When transcription is initiated, the splicing factors are suggested to be rapidly recruited from the speckles to nuclear regions where they are needed (Misteli, Caceres et al. 1997). The recruitment of splicing factors seems to be dependent upon transcription and protein phosphorylation, but other than that, little is known (Kuroyanagi, Onogi et al. 1998; Misteli and Spector 1999). Previous microscopy studies of adenovirus infected cells have shown that there is a dramatic reorganization of the nucleus during the intermediate phase of a lytic infection (Bridge and Pettersson 1995). At this stage, splicing factors are recruited to sites where viral genes are transcribed and processed (Bridge, Carmo-Fonseca et al. 1993). In this project, we investigated how the SR proteins, ASF/SF2 and SC35 are recruited to sites of transcription and splicing in adenovirus-infected cells using confocal microscopy.

2.1 Co-transcriptional RNA processing

This chapter will briefly summarize how proteins are expressed in eukaryotic cells, and describe the link between transcription, pre-mRNA splicing, and other RNA processes (see Fig. 1). Although capping, splicing and polyadenylation can occur as independent processes in vitro, recent studies indicate that most pre-mRNA processing occurs co-transcriptionally rather than post-transcriptionally in vivo (reviewed in (Daneholt 2001; Bentley 2002)). In this thesis, the term post-transcriptional refers to processes that occur after the RNA transcript has left the exit channel of the polymerase.

The DNA is transcribed in the nucleus by multi-subunit enzymes, the RNA polymerases (reviewed in (Lee and Young 2000)). In eukaryotic cells, there are three different RNA polymerases; I, II and III, which synthesise different classes of cellular RNA. RNA pol II transcribes mRNA and some small nuclear RNAs. All genes have a promoter area from which transcription of the gene starts. Most RNA pol II promoters contain the transcription start site, a TATA box, and DNA sequence elements to which regulatory transcription factors bind. The RNA pol II needs general transcription factors in order to assemble at the promoter and initiate transcription. After initiation, phosphorylation of the C-terminal domain (CTD) of RNA pol II switches the polymerase activity to elongation of transcription (reviewed in (Lee and Young 2000)).
When the pre-mRNA is about 25 nucleotides long, capping enzymes bound to the phosphorylated CTD of RNA pol II add the cap, an inverted methylated guanosine analogue, to the 5’ end. Only RNAs transcribed by pol II are capped at the 5’ end, and this is due to direct binding of the capping enzymes to pol II (reviewed in (Schroeder, Schwer et al. 2000) (Shatkin and Manley 2000)). The 5’ cap modification is important for protection against 5’ to 3’ exonucleases, and for recognition by the translation machinery through the cap-binding complex (CBC), and the translation initiation factor eIF4E (McKendrick, Thompson et al. 2001). The capping reaction can be further linked to the splicing process, as CBC also plays a role in splicing of the first intron and promotes the nucleocytoplasmic export of snRNAs (Izaurralde, Lewis et al. 1994; Izaurralde, Lewis et al. 1995).

Splicing of the new transcript can begin as soon as splice sites have been transcribed. A direct connection between RNA pol II transcription and splicing have been proposed but it

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Figure 1. mRNA biogenesis (adapted from (Dreyfuss, Kim et al. 2002)).
is still somewhat controversial (reviewed in (Hirose and Manley 2000; Neugebauer 2002)). In vitro splicing is stimulated by phosphorylated RNA pol II or by CTD alone ((Hirose, Tacke et al. 1999)) and different types of promoters appear to dispose a specific splicing pattern to transcripts initiated on the promoter ((Cramer, Pesce et al. 1997; Cramer, Caceres et al. 1999)). So far, the yeast U1snRNP component, Prp40 and the SR-related proteins called SCAF's have been shown to associate with the CTD ((Yuryev, Patturajan et al. 1996; Kim, Du et al. 1997)).

All eukaryotic mRNAs with the exception of histone mRNAs contain poly(A) tails. Polyadenylation occurs when the cleavage polyadenylation-specificity factor (CPSF), together with the downstream cleavage stimulatory factor (CstF) recognizes the poly(A) consensus sequence, AAUAAA, on the mRNA. Following endonucleolytic cleavage of the mRNA, poly(A) synthesis takes place. The formation of the poly(A) tail and the poly(A) tail itself are important for termination of transcription as well as for mRNA stability, splicing, transport from the nucleus to the cytoplasm and for translation (reviewed in (Zhao, Hyman et al. 1999)). Cleavage/polyadenylation factors have also been clearly associated with the CTD during transcriptional elongation (Hirose and Manley 2000). Polyadenylation and splicing appear to be connected events in the recognition of 3' terminal exon. For example, the poly(A) polymerase interacts with 65 kDa U2 snRNP Auxiliary Factor, U2AF65, and this interaction probably assists in exon recognition of terminal exons and promotes the assembly of the polyadenylation machinery within the exon (Vagner, Vagner et al. 2000).

RNA editing is a co- or post-transcriptional process that changes the nucleotide sequence of the substrate (Schaub and Keller 2002). RNA editing occurs in many organisms, and operates by many different molecular mechanisms; either by insertion or deletion of nucleotides or by base modification. Adenosine to inosine conversion catalysed by adenosine deaminases in pre-mRNA coding or non-coding regions (introns, untranslated regions) can alter the specificity of a codon or can affect splicing by altering splice-site sequences to produce alternative splicing (Rueter, Dawson et al. 1999).

During transcription, the pre-mRNA is bound not only by splicing factors but also by heterogeneous ribonucleoprotein particles (hnRNP's) (reviewed in (Kim and Dreyfus 2001)). These are thought to induce proper processing and folding of the pre-mRNAs. The hnRNP's remain bound after splicing is completed. Nucleo-cytoplasmic export occurs through the nuclear pore complex (NPC), which provides docking sites for transport complexes. During the export to the cytoplasm, some hnRNP's are removed at the NPC while others follow the mRNA to the cytoplasm and shuttle back to the nucleus.
The composition of the proteins bound to the mRNA is altered near the exon-exon junction following splicing. A protein complex is deposited, known as the exon-junction complex, (EJC), which serves as a binding platform for export factors (Zhou, Luo et al. 2000). This complex consists of at least DEK, SRm160, Y14 and RNPS1 (Kataoka, Yong et al. 2000; Le Hir, Izaurralde et al. 2000; Le Hir, Gatfield et al. 2001). Importantly, this complex provides a link between RNA splicing and export of the RNA. Three classes of factors appear to be involved in mRNA export; adapter proteins that bind directly to the mRNA, receptor proteins that recognize and bind to adapter proteins, and NPC components that mediate the export across the nuclear membrane (reviewed in (Komeili and O'Shea 2001)). So far, two pathways for export of mRNAs have been uncovered, involving two different export receptors, CRM1 and TAP (reviewed in (Cullen 2000)). In one of the pathways, an RNA binding adaptor protein, REF (RNA and export factor-binding protein), interacts directly with the export receptor, TAP (Kang and Cullen 1999) and is recruited to mRNA sites near exon-junctions as part of the EJC (Fig. 1) (Le Hir, Gatfield et al. 2001). At the EJC, REF becomes bound to the spliced mRNA, which is then efficiently exported to the cytoplasm. REF is most efficiently recruited to mRNA during splicing (Luo and Reed 1999).

After reaching the cytoplasm, the mRNA is translated into a protein by the ribosome, a multi-component complex consisting of RNA and proteins. The mRNAs in the cytoplasm are eventually subjected to degradation (reviewed in (Guhaniyogi and Brewer 2001)). The poly(A) tail is gradually shortened, which in turn causes decapping at the 5′ end followed by a 5′ to 3′-exonucleolytic degradation. RNA stability can also be regulated through AU-rich elements present in the 3′ untranslated regions of short-lived mRNAs.

Even if mRNAs are exported to the cytoplasm, they may be defective, due to incomplete splicing or splicing errors. These defective mRNAs often contain premature translation termination stop codons, which trigger their rapid degradation by a process termed nonsense-mediated mRNA decay (also called mRNA surveillance) (Kim and Dreyfus 2001).
3. Introduction to pre-mRNA splicing

3.1 The mechanism of pre-mRNA splicing

Most newly synthesised mRNAs contain stretches of non-coding RNA (introns) that must be excised before the RNA can be translated into a protein (see Fig. 2). The introns are removed from the pre-mRNA by a large macromolecular complex called the spliceosome. The consensus sequence elements at the splice sites of the transcript direct the assembly of the spliceosome. In mammals, the 5´ splice site signal is AG/GURAGU (exon/intron), (R = purine). At the 3´ splice site, three different signals are used; the branch point sequence (BPS), YNYURAC, (Y = pyrimidine, N = variable nucleotides), a polypyrimidine tract (10-20 nucleotides) and the actual 3´ splice site, YAG/N (intron/exon) (Burge, Tuschi et al. 1999). There is also a minor class of introns that have dinucleotides AU and AC at their 5´ and 3´ splice site and they are most often spliced by the minor spliceosome (Tarn and Steitz 1996).

![Pre-mRNA with classical splicing signals](image)

Figure 2. Pre-mRNA with the classical splicing signals found in the major class of introns.

The specificity of the splicing reaction is also dependent on exonic or intronic enhancer and repressor elements (reviewed in (Blencowe 2000; Smith and Valcarcel 2000; Hastings and Krainer 2001)). Many transcripts have multiple consensus splice site sequences whose activities are regulated by transacting factors binding to the enhancer or repressor element. Alternative choices of splice sites and alternative splicing creates different transcripts and subsequently, multiple protein products (see example in Fig. 3) (reviewed in (Lopez 1998)). Alternative splicing is perhaps the most important mechanism for increasing protein diversity in vertebrates (reviewed in (Graveley 2001)).
3.2 The chemistry of the splicing reaction

The pre-mRNA is spliced by two trans-esterification reactions (see Fig. 4). In the first, reaction the 2’ hydroxyl group of the conserved A residue in the BPS of the intron attacks the 3’-5’ phosphodiester bond at the 5’ splice site. This results in cleavage at the 5’ exon-intron junction and generation of two splicing intermediates; a 5’ exon with a free hydroxyl group at its 3’ terminus, and the lariat intermediate with the 5’ end of the intron joined with the branch site adenosine via a 5’-2’ bond. In the second reaction, the free hydroxyl group of the 5’ exon attacks the 3’-5’ phosphodiester bond at the 3’ splice site and displaces the intron lariat with a 3’ hydroxyl group. This results in the joining of the two exons via a 5’-3’ phosphodiester bond generating the spliced product (reviewed in (Burge, Tuschl et al. 1999)).

Figure 4. The catalytic steps in pre-mRNA splicing (adapted from (Gesteland 1999)).
3.3 Components of the splicing machinery

The spliceosome

In the major spliceosome, five small nuclear RNAs participate in the splicing reaction; U1, U2, U4, U5 and U6 RNAs. These RNAs vary in length from 100 to 200 nucleotides and associate with proteins to form small nuclear ribonucleoprotein particles (snRNPs) (reviewed in (Will and Luhrmann 2001)). The RNA and protein components of snRNPs have essential functions in recognition, selection and regulation of spliceosome assembly and catalysis. The minor spliceosome uses the same biochemical splicing mechanism as the major spliceosome but consists of a different set of snRNPs; U11, U12, U4atac, U6atac and U5 (reviewed in (Wu and Krainer 1999)). The proportion of introns that are spliced by the minor (U12) spliceosome is only about one in a thousand. This thesis will focus on splicing by the major (U2) spliceosome.

The snRNP biogenesis

All U snRNAs, with the exception of U6 and U6atac, are transcribed by RNA pol II, capped and exported to the cytoplasm where the U snRNP assembly is initiated (reviewed in (Will and Luhrmann 2001)). The export of the capped U snRNAs requires an export adapter, PHAX (phosphorylated adapter for RNA export), to mediate the interaction with the export receptor CRM1/RanGTP (Ohno, Segref et al. 2000). In the cytoplasm, the U snRNA interacts in an ordered, stepwise manner with seven Sm proteins. The Sm proteins interact as pre-formed complexes with the Sm site of the U snRNA. After methylation of the cap and 3´ end trimming, the U snRNP Sm core is imported to the nucleus. The methylated cap and the Sm core domain form a U snRNP nuclear localization signal (NLS) required for the import of the U snRNP. Import is mediated by the import factor, Snurportin-1, which binds to the methylated cap on the snRNAs and to the general import factor, Importin-β.

After import, these factors dissociate and the specific proteins for each U snRNP, which appear to be imported separately, associate with the U snRNP Sm core. Biogenesis of the U6 snRNP, and presumably the U6atac snRNP differ from that of the other U snRNPs. The U6 snRNA is transcribed by RNA pol III and assembly of the U6 snRNP is thought to take place entirely in the nucleus.
The SR protein family of splicing factors

The SR proteins are a family of highly conserved serine/arginine-rich RNA binding proteins. They are essential for constitutive splicing and regulate alternative splice site selection in a concentration-dependent manner both in vivo and in vitro (Ge and Manley 1990; Krainer, Conway et al. 1990; Zahler, Lane et al. 1992; Zahler, Neugebauer et al. 1993; Caceres, Stamm et al. 1994). Some SR protein functions are shared by all members, but some functions are specific to certain family members (reviewed in (Tacke and Manley 1999; Graveley 2000). SR proteins have been identified in all metazoan species examined, and the family currently contains 10 members (see Fig. 5). Any member of the SR protein family can complement splicing-deficient S100 extracts (cytoplasmic extracts from HeLa cells that contain all components necessary for in vitro splicing except a functional SR protein) (reviewed in (Fu 1995)).

| SRp20 | RRM | RS |
| SC35 | RRM | RS |
| 9G8 | RRM | RS |
| SRp46 | RRM | RS |
| SRp54 | RRM | RS |
| SRp30c | RRM | RRM | RS |
| ASF/SF2 | RRM | RRM | RS |
| SRp40 | RRM | RRM | RS |
| SRp55 | RRM | RRM | RS |
| SRp75 | RRM | RRM | RS |

Figure 5. The human SR proteins (Graveley 2000).

The SR proteins have a modular structure with an N-terminal RNA binding domain consisting of either one or two RRMs (RNA Recognition Motifs) and a C-terminal domain containing repetitive arginine-serine dipeptides, a so called RS domain. The RNA binding domain provides specificity for binding certain mRNAs, while the RS domain interacts with other proteins (Wu and Maniatis 1993; Kohtz, Jamison et al. 1994; Xiao and Manley 1997) (Caceres, Misteli et al. 1997; Mayeda, Screaton et al. 1999). The RNA binding domain can be exchanged between SR proteins and can bind RNA in the absence of the RS domain.
(Chandler, Mayeda et al. 1997; Mayeda, Screaton et al. 1999). The RS domain can also be functionally exchanged between SR proteins and it can even function when fused to a heterologous RNA-binding domain (Chandler, Mayeda et al. 1997; Graveley and Maniatis 1998; Wang, Xiao et al. 1998).

The SR proteins are phosphorylated at serine residues within the RS domain and their activity is thus regulated by this phosphorylation (Kanopka, Muhlemann et al. 1998; Wang, Lin et al. 1998), (Xiao and Manley 1997; Xiao and Manley 1998). The two most extensively studied protein kinases that have been shown to phosphorylate SR proteins are SRPK1 and Clk/Sty (Gui, Tronchere et al. 1994; Colwill, Feng et al. 1996). Phosphorylated SR proteins are required for the assembly of the spliceosome and dephosphorylation of the same proteins are required for catalysis of splicing in vitro (Cao, Jamison et al. 1997). However, both hyper- and hypophosphorylation of SR proteins can reduce their splicing activity (Kanopka, Muhlemann et al. 1998; Prasad, Colwill et al. 1999).

SR proteins are ubiquitously expressed, but there are cell-type specific differences in abundance. This suggests that different cell types might have a distinct pattern of relative SR protein concentrations, which could influence regulation of alternative splicing (Zahler, Neugebauer et al. 1993; Screaton, Caceres et al. 1995). The function of SR proteins in alternative 5’splice site selection can be antagonized by the heterogeneous nuclear ribonucleoprotein particle A1, hnRNPA1 (Eperon, Makarova et al. 2000). hnRNPA1 can prevent binding of ASF/SF2 to enhancer elements in a concentration dependent manner (Mayeda and Krainer 1992).

Only a few of the SR proteins in metazoans have been shown to be essential. SRp55 is essential for proper development in Drosophila (Ring and Lis 1994) and ASF/SF2 (Alternative Splicing Factor/Splicing Factor 2) is essential for viability of chicken DT40 cells (Wang, Takagaki et al. 1996). When the ASF/SF2 homologue, rsp-3 in C.elegans was targeted by RNAi, an embryonic lethal phenotype was observed (Longman, Johnstone et al. 2000). However, individual silencing of six other SR protein-encoding genes had no effect on embryonic development, although simultaneous silencing of all six was lethal (Longman, Johnstone et al. 2000). The reason why only some of the SR proteins are essential is unknown, either, the non-essential SR proteins do not participate in splicing of essential genes or, alternatively, other SR proteins functionally substitute for the missing protein.
There are also a number of SR proteins distinct from the classical SR proteins that are required for splicing. These are collectively referred to as SR-related proteins, SRrps (reviewed in Blencowe, Bowman et al. 1999). The SRrps have an RS domain but not necessarily an RNA binding domain. Although many of them are essential splicing factors they cannot complement splicing-deficient S100 extracts. Examples include both subunits of U2AF (Zhang, Zamore et al. 1992) (Zamore, Patton et al. 1992), snRNP components (like U1-70K (Spritz, Strunk et al. 1987)), splicing regulators (like hTra2 (Dauwalder, Amaya-Manzanares et al. 1996)), splicing co activators (like SRm160 (Blencowe B.J. 2000)), RNA helicases (like hPrp16 (Zhou and Reed 1998)) and protein kinases (like Ctk/Sty (Johnson and Smith 1991)).

One of the SR-related proteins, SRp38 (also known as NSSR-1 (Komatsu, Kominami et al. 1999), TASR-2 (Yang, Embree et al. 2000) or SRrp40 (Cowper, Caceres et al. 2001)), has a typical SR protein structure but very unusual properties. Phosphorylated SRp38, cannot activate splicing and is essentially inactive in splicing assays. However, dephosphorylation converts SRp38 to a potent general repressor of splicing (Shin and Manley 2002) and cell cycle-specific dephosphorylation of SRp38 has been proposed to play a role in gene silencing during mitosis.

### 3.4 The splicing process

During the stepwise assembly of the spliceosome, the splicing reaction takes place in two catalytic steps (see Fig. 6) (reviewed in Hastings and Krainer 2001) (Reed 2000)). The first complex formed in this pathway is the E-complex.

(i), Formation of the E-complex, also called the commitment complex, involves the initial recognition of splice sites and commits the pre-mRNA to spliceosome assembly and splicing. Thus, assembly of the E-complex is likely to be the key step at which alternative splicing is regulated. It is initiated by U1 snRNP binding to the 5’ splice site by direct base pairing of U1 snRNA, SF1 (Splicing Factor 1) binding to the adenosine nucleotide within the BPS, 65 kDa U2AF binding to the polypyrimidine tract and 35 kDa U2AF to the AG dinucleotide at the 3’ splice site (Gaur, Valcarcel et al. 1995; Berglund, Abovich et al. 1998) (Eperon, Ireland et al. 1993). SF1 directs the spliceosome to the intron, and when SF1 leaves the BPS, it assists in the base pairing of U2 snRNP to the same sequence. U2AF promotes the binding of U2 snRNP to the BPS by direct interaction between the 65 kDa subunit and SAP155, a component of U2 snRNP (Ruskin, Zamore et al. 1988). SR proteins also promote
assembly of the E-complex by assisting binding of both the U1 70K protein of U1 snRNP and U2AF35, thus contributing to the bridging of the splice sites at early stages of the reaction (reviewed in (Graveley, Hertel et al. 2001). Selection of 3´ splice sites can be AG-dependent or AG-independent. The 35 kDa U2AF is essential for splicing of AG-dependent introns which require the AG dinucleotide for spliceosome assembly, but dispensible for AG-independent introns, which have an extended pyrimidine tract (Wu, Romfo et al. 1999).

(ii), U2 snRNP is believed to bind loosely to the BPS in the E-complex through its subunits, SF3a and SF3b. During the subsequent formation of the A-complex, an ATP-dependent process leads to stable U2 snRNP binding to the BPS (Das, Zhou et al. 2000). The stable U2 snRNP binding require U2AF65-associated DEAH helicase, UAP56 (U2AF65 associated protein of 56 kDa) which is believed to facilitate the RNA-RNA rearrangements (Fleckner, Zhang et al. 1997).

(iii), Subsequent binding of U5-U4/U6 allows the transition from A-complex to B-complex. U5 binds to sequences in both the 5´ and 3´ exons and base pairs with the 5´ end of U1 snRNA. These interactions bring the two splice sites together.

(iii), The last complex, the C-complex, is formed when U6 snRNP dissociates from U4, forms base pairing with U2 snRNP and replaces U1 snRNP as the factor interacting with the 5´ end of the intron (reviewed in (Murray and Jarrell 1999)). It is believed that RNA rearrangements activate the spliceosome for catalysis of splicing. The binding of U2 to U6 snRNA, positions the branch point and the 5´ splice site sequence for the first catalytic step, generating the lariat intermediate and the free 5´ exon. Before the second catalytic step, the spliceosome undergoes additional conformational changes, creating new RNA-RNA interactions. The U2-U6 snRNP interaction is rearranged, and it makes contact with the 5´ splice site in the intron-lariat. Finally, the 5´ splice site in the free exon is joined together with the 3´ exon of the lariat intermediate (Burge, Tuschl et al. 1999).

The splicing factor, hSlu7 is required to hold exon 1 tightly within the spliceosome in order to select the correct AG at the 3´ splice site (Chua and Reed 1999). All steps in the spliceosome assembly require ATP hydrolysis, except formation of the E-complex. It is believed that the spliceosome consumes ATP to rearrange RNA-RNA and RNA-protein interactions during assembly and the catalytic steps of splicing (reviewed in (Staley and Guthrie 1998)).
Figure 6. The spliceosome assembly.
3.5 Exon definition

The average vertebrate gene consists of multiple internal exons (usually 300 nucleotides or less) separated by introns that are considerably larger (possibly mega bases in length). Berget and co-workers observed that a 5´ splice site on the downstream side of an exon promotes splicing of the intron immediately upstream and proposed that initial recognition requires an interaction between 5´ and 3´ splice sites across the short exon (<350 nucleotides) (see Fig. 7) (Berget 1995). In this exon definition model, the binding of the U1 and U2 snRNPs and associated factors, including U2AF65, U2AF35 and SR proteins, define the exon. In the case of terminal exons, it has been shown that the cap structure is essential for the recognition of the first exon (Izaurralde, Lewis et al. 1994). Terminal exons that end with a poly(A) site use polyadenylation factors for interactions across 3´ terminal exons (Berget 1995). More recently, Lam et al demonstrated that a single exonic splicing enhancer element, ESE promotes the recognition of both exon/intron junctions within the same step during exon definition. They suggested that the exonic splicing enhancer element recruits a multi-component complex that contains the minimal complement of components of the splicing machinery required for 5´ and 3´ splice site selection (Lam and Hertel 2002).

Figure 7. The exon definition model.

3.6 Splicing enhancers and silencers

Pre-mRNA splicing enhancers play a critical role in the regulation of alternative splicing and in correct splice site recognition of constitutively spliced pre-mRNAs elements (reviewed in (Blencowe 2000; Smith and Valcarcel 2000; Hastings and Krainer 2001)). Exonic splicing enhancers, ESEs are required for excision of introns that contain splice sites that do not conform to the consensus sequence (reviewed in (Varani G. 1998)). Although splicing enhancers are usually located downstream of the affected introns, they are also found within introns and upstream of regulated 5´ splice sites (Hastings, Wilson et al. 2001). Many of these RNA elements are recognized by SR proteins, which are thought to function by recruiting components of the general splicing machinery to the nearby splice sites (Sun, Mayeda et al.
1993; Liu, Zhang et al. 1998). The strength of splicing enhancers is determined by the relative activities of the bound splicing factor, the number of proteins within the enhancer complex and the distance between the enhancer and the intron (Graveley, Hertel et al. 1998a). The enhancer elements are usually positioned less than 70 nucleotides from the 3´ splice site. There is also increasing evidence that exons contain sequences that inhibit splicing, exonic splicing silencers (Graveley, Hertel et al. 1998a). In at least some cases, silencing is mediated by hnRNP proteins (heterogeneous nuclear ribonucleoproteins), such as hnRNPA/B and hnRNP H (Del Gatto-Konczak, Olive et al. 1999). In vivo, hnRNP proteins are thought to bind co-operatively to mRNA and form stable hnRNP complexes that in some cases can compete with spliceosome assembly and with enhancer-bound SR proteins (Eperon, Makarova et al. 2000; Zhu, Mayeda et al. 2001).

SR proteins may also mediate inhibition through the splicing silencers. Kanopka et al found that SR proteins inhibit splicing of the regulated adenovirus L1 pre-mRNA by binding an intronic repressor element, located immediately upstream of the IIIa branch site and thereby preventing U2 snRNP recruitment to the spliceosome. They also demonstrated that moving the IIIa repressor element to the second exon of the IIIa pre-mRNA converted the repressor element to a classical splicing enhancer element (Kanopka, Muhle mann et al. 1996). Thus, depending on where they bind (in the intron or exon), SR proteins may act as splicing enhancers or silencers.

3.7 Organization of splicing factors in the cell nucleus

The splicing factors are diffusely distributed throughout the nucleus, with enrichment in compartments commonly referred to as speckles (recently reviewed in (Misteli 2000; Lamond and Spector 2003)). At the fluorescence-microscopic level, the speckles appear as irregular, punctuated structures, which vary in size and shape and when examined by electron microscopy they are seen as clusters of interchromatin granules (Thiry 1995).

Originally, speckles were characterized as sites having high concentrations of splicing factors but more recently they have also been shown to contain transcription factors, 3´ processing factors and ribosomal proteins (Mortillaro, Blencowe et al. 1996; Schul, van Driel et al. 1998; Mintz, Patterson et al. 1999; Dostie, Lejbkowicz et al. 2000). They are often located close to highly transcribed genes but are not themselves sites of active transcription or splicing (Misteli, Caceres et al. 1997; Cmarko, Verschure et al. 1999). Although several proteins with possible structural roles in the nucleus, such as lamin A and snRNP-associated
actin, have been found in the speckles, no underlying scaffold has so far been identified (Nakayasu and Ueda 1984; Jagatheesan, Thanumalayan et al. 1999).

One function of the speckles is thought to be recycling and/or acting as a temporal storage and assembly site for splicing factors, but since they contain other RNA processing factors and accumulate polyadenylated RNA (Carter, Taneja et al. 1991; Visa, Puvion-Dutilleul et al. 1993), there are reasons to believe that the speckles could have more functions. The speckles consist of numerous spherical sub domains referred to as sub speckles. Each speckle is composed of 5-50 sub speckles. Spector and co-workers have proposed that the compartmentalization into sub speckles may represent an efficient way of organizing these factors for their subsequent transport to transcription/RNA processing sites (Mintz and Spector 2000).

Splicing factors are also found in other nuclear structures such as Cajal bodies, interchromatin-associated zones and perichromatin fibrils. The Cajal bodies contain newly assembled snRNPs and small nucleolar ribonucleoproteins (snoRNPs), the latter being active in ribosomal RNA processing. Recent evidence suggests that Cajal bodies may be involved in coordinating the assembly and maturation of nuclear RNP and possibly other macromolecular complexes (reviewed in (Ogg and Lamond 2002)). The interchromatin-associated zones are found adjacent to speckles and contain U1 snRNA (Visa, Puvion-Dutilleul et al. 1993). The perichromatin fibrils can be observed by electron microscopy and are believed to represent nascent transcripts (Fakan 1994).

The majority of pre-mRNA splicing probably occurs co-transcriptionally at the site of transcription (Beyer and Osheim 1991; Xing, Johnson et al. 1993; Bauren and Wieslander 1994). The pre-mRNA is spliced, released from the site of transcription and exported to the cytoplasm for translation. Transcripts that are not correctly spliced or cleaved at their 3´-ends, accumulate at the site of transcription indicating that completion of splicing and 3´-end processing is necessary for the release of transcripts (Custodio, Carmo-Fonseca et al. 1999). However, highly expressed pre-mRNAs or pre-mRNAs with many introns, like the RNA for the Balbiani ring of Chironomous tentants can be spliced after its release. Wetterberg et al analyzed the splicing in the Balbiani Ring 3, (BR3), gene in Chironomous tentants and showed in vivo that intron excision initiates co-transcriptionally in an overall 5´ to 3´ order. The proximal 5´ introns of the BR3 transcript are excised co-transcriptionally while the most distal 3´ introns are excised post-transcriptionally (Wetterberg, Bauren et al. 1996). This suggests that complete splicing is not crucial for the BR3 mRNA to leave the site of
transcription, and that the timing of the release of the transcript depends more on the extent of splicing that is necessary.

Changes in the overall transcriptional activity of a cell cause redistribution of splicing factors. Inhibition of transcription results in immobilization of the splicing factors in enlarged speckles (Misteli, Caceres et al. 1997). On the other hand, when transcription levels are high during an adenovirus infection, the majority of the splicing factors are redistributed to viral replication and transcription centres, where they accumulate in ring-like structures (Bridge and Pettersson 1995). In contrast, during infection with herpes simplex virus, which only has four genes containing introns, no obvious recruitment occurs, which suggests that the accumulation of splicing factors at sites of active transcription is intron-dependent (Phelan, Carmo-Fonseca et al. 1993). Similar results have been observed when transcribing artificially intron-less genes (Huang and Spector 1996).

Several protein kinases and protein phosphatases are also localized to speckles (Colwill, Pawson et al. 1996; Mintz, Patterson et al. 1999; Brede, Solheim et al. 2002). Dephosphorylation is perhaps the major control mechanism for localization of at least the SR protein family of splicing factors to speckles. Time-lapse microscopy experiments in living cells show that release of splicing factors from speckles is inhibited in the presence of a kinase inhibitor (Misteli, Caceres et al. 1997). Reassociation of splicing factors in speckles requires the removal of one or more phosphate groups (Misteli and Spector 1996). This supports the idea that speckles might be involved in regulating the pool of factors that are accessible to the transcription/RNA processing machinery. Controlling the concentration of splicing factors in the nucleoplasm might optimise the efficiency of splicing.
4. Herpes Simplex virus-1

4.1 Structure and genome organisation
Herpes simplex virus-1 (HSV-1) is a member of the *Herpesviridae* family. Herpes viruses infect members of all groups of vertebrates and the same host can be infected with multiple distinct types. To date, eight human herpes viruses have been described and each causes a characteristic disease. HSV-1, for instance, causes oral cold sores and ocular lesions whereas infections by two other human herpes viruses, Epstein-Barr virus and human herpes virus-8, are linked to human cancer (reviewed in (Roizman 1996) (S.J Flint 2000)).

HSV-1 is a double stranded DNA virus with an icosahedral capsid surrounded by an envelope. The virions of the herpes viruses, contain far more proteins than any other virus described so far. Over half of the proteins encoded by the HSV genome are present in the virion. The virion proteins are found in the nucleocapsid, in the layer between the envelope and the nucleocapsid (the tegument layer) and embedded in the envelope. The diameter of the virion is approximately 200 nm. The linear genome is organised into two unique segments; one long region of 126 kb (UL) and one short region of 26 kb (US). Both the long and the short region have inverted repeat sequences and terminal repeats. The HSV-1 can recombine via the inverted repeats. The viral genome contains three origins of replication, one within the UL and two within the US (reviewed in (Roizman 1996) (S.J Flint 2000)).

4.2 Herpes simplex virus-1 infection
HSV-1 infection occurs via skin or mucosa. The virus starts a primary replication in the infected cells immediately after infection. During this phase, the virus travels via an axonal transport mechanism into the nervous system and infects peripheral neurones. In the neurons, the virus establishes a latent infection and hides in dorsal root ganglia. The nervous system is an ideal hiding site for the virus since it is not patrolled by the immune system. However, the major factor for latency is a restricted gene expression (reviewed in (Whitley 1996) (S.J Flint 2000)).

During a latent infection, normal viral transcription is blocked by an unknown mechanism. The terminally differentiated neurones neither replicate nor divide, so once a viral genome is established, it does not need to replicate to persist for the life of the neuron. When reactivated, HSV-1 travels down the sensory nerves to mucosal surfaces where it restarts the lytic life cycle, which causes the pathologic manifestation of cold sores. It is not
clear what causes the reactivation but there are many possible alternatives, including both physical and psychological factors e.g. sunburn, stress and nerve damage. Some individuals experience reactivation every two to three weeks while others experience no reactivation (reviewed in (Whitley 1996; S.J Flint 2000)).

4.3 Herpes simplex virus-1 lytic life cycle

Herpes simplex virions attach to the cell when the viral envelope proteins bind to cellular receptors, such as heparan sulphate, and to the extra cellular matrix. Thereafter, the virion enters the cell by fusion of the viral envelope with the plasma membrane. The tegument proteins are released into the cytoplasm and the uncoated virion is transported via microtubules to the nucleus, where it docks at the nuclear pore and releases the viral DNA into the nucleus. Some of the tegument proteins like vhs (virion host cell shut-off factor) stay and act in the cytoplasm, while others are transported into the nucleus (reviewed in (Roizman 1996) (S.J Flint 2000).

During a productive infection, the HSV-1 genes are expressed in a temporal cascade. After expression of the immediate-early genes (IE), the IE-gene products, in turn activates transcription of the early genes (E) and auto regulate transcription of IE genes. The E proteins function mainly in viral DNA replication and the production of substrates for DNA synthesis. When sufficient levels of the viral replication proteins have accumulated in the cell, the replication of viral genome starts and the expression of the late genes (L) starts. L proteins are primarily structural proteins of the virion and proteins needed for virus assembly (reviewed in (Weir 2001).

Newly replicated viral DNA is packaged into preformed capsids in the nucleus. Some tegument proteins are added in the nucleus and others in the cytoplasm. It is not known if the membrane with embedded glycoproteins of mature virions originates from the inner nuclear membrane or are derived from the Golgi or the endoplasmatic reticulum. How the virion is subsequently transported to the plasma membrane is also currently unknown. The enveloped virion can either remain cell-associated or it can be released from the cell by exocytosis for re-infection (reviewed in (Roizman 1996; S.J Flint 2000).
5. The multifunctional HSV-1 protein, ICP27

Herpes simplex virus-1 infected cell protein, ICP27 is a 63 kDa phosphoprotein, which is essential for lytic infection and is the only IE protein that is conserved among all known herpes viruses. ICP27 is a multifunctional protein: it regulates expression of both viral and cellular genes at both the transcriptional and post-transcriptional levels (reviewed in (Clements 1998)).

5.1 ICP27-interacting proteins

ICP27 has been shown to interact with a number of proteins (Table 1).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hnRNP K</td>
<td>transcription activator, regulate translation</td>
<td>yeast-two hybrid screen</td>
<td>(Wadd, Bryant et al. 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in vivo binding studies</td>
<td></td>
</tr>
<tr>
<td>casein kinase 2</td>
<td>serine/threonine protein kinase</td>
<td>yeast-two hybrid screen</td>
<td>(Wadd, Bryant et al. 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in vivo binding studies</td>
<td></td>
</tr>
<tr>
<td>common Sm proteins</td>
<td>protein components of snRNP</td>
<td>in vivo binding studies</td>
<td>(Sandri-Goldin and Hibbard 1996)</td>
</tr>
<tr>
<td>SAP 145</td>
<td>protein component of U2 snRNP</td>
<td>yeast-two hybrid screen</td>
<td>(Bryant, Wadd et al. 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in vivo binding studies</td>
<td></td>
</tr>
<tr>
<td>TAP</td>
<td>cellular export receptor</td>
<td>yeast-two hybrid screen</td>
<td>(Chen, Sciabica et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in vivo binding studies</td>
<td></td>
</tr>
<tr>
<td>REF</td>
<td>cellular RNA export factor</td>
<td>yeast-two hybrid screen</td>
<td>(Koffa, Clements et al. 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in vivo binding studies</td>
<td></td>
</tr>
<tr>
<td>RNA pol II holoenzyme</td>
<td>catalyses synthesis of RNA</td>
<td>in vivo binding studies</td>
<td>(Zhou and Knipe 2002)</td>
</tr>
<tr>
<td>P32</td>
<td>Cellular (mainly mitochondrial ) multifunctional protein</td>
<td>yeast-two hybrid screen</td>
<td>(Bryant, Matthews et al. 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in vivo binding studies</td>
<td></td>
</tr>
<tr>
<td>SRp20</td>
<td>member of the SR protein family of splicing factors</td>
<td>yeast-two hybrid screen</td>
<td>(Sciabica, Dai et al. 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>in vivo binding studies</td>
<td></td>
</tr>
<tr>
<td>SRPK1</td>
<td>SR protein kinase 1, highly specific for serine/arginine dipeptides</td>
<td>in vivo binding studies</td>
<td>(Sciabica, Dai et al. 2003)</td>
</tr>
</tbody>
</table>
5.2 Functional domains within ICP27

ICP27 is an RNA binding protein that shuttles between the nucleus and the cytoplasm (see Fig. 9). It has a nuclear export signal (Sandri-Goldin 1998) and nuclear and nucleolar localization signals (Mears, Lam et al. 1995), that may control its sub-cellular localization.

The C-terminus of ICP27 is the most conserved part of the protein and is important for its repressing and activating functions in viral transcription (Hardwicke, Vaughan et al. 1989; Rice and Knipe 1990; Rice and Lam 1994). The extreme C-terminus of ICP27 contains an Sm-homology domain, a structure that mediates protein-protein interactions between Sm proteins associated with U snRNAs (Sandri-Goldin and Hibbard 1996). The amino-terminal acidic region of ICP27 performs essential functions in viral DNA replication (Rice, Lam et al. 1993).

The structural domains in ICP27 involved in RNA binding are the RGG-box (Mears and Rice 1996), and the KH-like domains, KH1, KH2 and KH3 (Soliman and Silverstein 2000). The RGG box is an arginine- and glycine-rich region similar to an RNA binding motif found in a number of cellular proteins that are involved in nuclear RNA processing (Lengyel, Guy et al. 2002). The KH-like domains bind single stranded RNA individually or in combination. ICP27 has been shown to interact with a broad range of HSV-1 mRNAs through its RGG box (Sokolowski, Scott et al. 2003).

Figure 8. Schematic representation of the ICP27 protein (not to scale), (adapted from (Bryant, Wadd et al. 2001)).
6. The role of ICP27 at the post-transcriptional level

6.1 ICP27 promotes RNA 3’ end processing at weak viral poly(A) sites

There are several well-documented examples in both mammalian and viral systems that employ alternative or regulated usage of poly(A) sites as a mean of controlling gene expression (Galli, Guise et al. 1988; Falck-Pedersen and Logan 1989). Poly(A) site usage is dependent on the inherent poly(A) site efficiency and/or on the concentrations of components of the 3’ processing complex. Some HSV-1 late mRNAs contain poly(A) addition signals that function poorly in the absence of ICP27 (McLauchlan, Simpson et al. 1989; McLauchlan, Phelan et al. 1992). UV cross linking experiments have shown that ICP27 enhances binding of protein factors, including the 64 kDa subunit of CstF, to poly(A) sites of viral mRNAs from viral mRNAs with weak poly(A) signals (McGregor, Phelan et al. 1996). The L poly(A) sites, that responded to ICP27 were inherently less efficient than IE and E sites. This indicates that their poly(A) site strength and their enhanced expression in the presence of ICP27 may influence the temporal expression of the L genes. Whether cellular poly(A) site usage is affected during an HSV infection is still unknown.

6.2 ICP27 stabilizes and binds to 3’ ends of labile mRNA

The intrinsic lifetime of an mRNA can be a critical parameter in the regulation of gene expression. Within the 3’ untranslated regions of many mRNAs there are AU-rich sequences that signal rapid turnover of the mRNA. Some proteins stabilize such mRNAs by binding to these sequences whereas other factors can induce shortening of the poly(A) tail (reviewed in (S.J Flint 2000)). ICP27 has been shown to bind and stabilize labile 3’ ends of mRNAs (Brown, Nakamura et al. 1995). ICP27 was, by itself, able to increase the half-life and stimulate the steady-state accumulation of reporter genes containing AU-rich untranslated regions. However, the mechanism behind this stimulation is still unknown.

6.3 ICP27 stimulates the nuclear to cytoplasmic export of intronless viral mRNAs

All mRNAs made in the nucleus must be transported to the cytoplasm for translation. Cellular pre-mRNAs that contain introns and splice sites are ordinarily retained in the nucleus until they are completely spliced or degraded. Koffa et al recently demonstrated that ICP27 interacts with REF in order to stimulate the export of intronless viral mRNAs (Koffa, Clements et al. 2001). Shortly after, Sandri-Goldin and co-workers confirmed that ICP27
interacts with REF for export of intronless viral mRNAs (Chen, Sciabica et al. 2002). They also found an \textit{in vivo} interaction between ICP27 and TAP but not with CRM1, demonstrating that ICP27 interacts with REF to direct HSV-1 intronless mRNAs to the TAP export pathway.

6.4 ICP27 inhibits pre-mRNA splicing
In 1987, Martin et al demonstrated that an HSV-1 infection caused a redistribution of snRNPs from a normal widespread speckled pattern to a highly punctuated organization in the nucleus (Martin, Barghusen et al. 1987). It has subsequently been shown that ICP27 is necessary for this event (Sandri-Goldin and Mendoza 1992; Phelan, Carmo-Fonseca et al. 1993; Hardy and Sandri-Goldin 1994; Sandri-Goldin, Hibbard et al. 1995). Redistribution of splicing factors like snRNP has also been shown during infection with adenovirus and influenza virus and may reflect movement of the factors from active sites of splicing to inactive storage sites (Bridge, Carmo-Fonseca et al. 1993; Fortes, Lamond et al. 1995). ICP27 was shown to colocalize with the redistributed snRNPs at later time points of infection (Phelan, Carmo-Fonseca et al. 1993) and Sandri-Goldin et al also demonstrated a physical interaction between ICP27 and U1 snRNP (Sandri-Goldin and Hibbard 1996). In the same study it was shown that pre-mRNAs with introns accumulated in the nucleus during wild type HSV infections but not in cells infected with a mutant virus lacking ICP27. In a separate paper, it was shown how splicing of a \(\beta\)-globin transcript was inhibited in nuclear extracts prepared from HSV-1 wild-type infected cells but not from cells infected with a mutant virus lacking ICP27 (Sandri-Goldin 1994). These results suggested that ICP27 has a splicing inhibitory function, but it still remained to be elucidated whether ICP27 is directly involved, or if another viral protein whose production or activity is dependent on ICP27, causes the inhibition. More studies on ICP27-induced splicing inhibition are presented and discussed in the section \textit{Present investigation}.

6.5 ICP27 homologues in other herpes viruses
ICP27 homologues have been identified in each of the herpes virus subfamilies (listed in Table 2). The functional data that are available for ICP27 homologues indicate that although these proteins show sequence similarity, they exhibit considerable functional diversity. For example, the human cytomegalovirus (CMV) UL69 and varicella-zoster virus (VZV) ORF4 proteins primarily stimulate gene expression at the level of transcription (Moriuchi, Moriuchi et al. 1994; Winkler, Rice et al. 1994; Defechereux, Debrus et al. 1997). In contrast, functional studies of the Epstein-Barr virus (EBV) SM protein, herpes virus samiri (HVS)
ORF57, and human herpesvirus-8 (HHV-8) ORF57, indicate that these proteins function at post-transcriptional steps of gene expression and that they shuttle between the nucleus and the cytoplasm (Bello, Davison et al. 1999; Boyle, Ruvolo et al. 1999; Goodwin, Hall et al. 1999; Gupta, Ruvolo et al. 2000; Kirshner, Lukac et al. 2000). The EBV SM protein activates expression of intronless reporter gene constructs and inhibits expression of intron-containing constructs (Ruvolo, Wang et al. 1998). SM also stimulates viral gene expression by enhancing levels of cytoplasmic and nuclear EBV mRNAs and by promoting EBV mRNA export (Semmes, Chen et al. 1998). Further, Boyer et al have shown that the SM protein has the ability to stimulate growth of an HSV-1 ICP27-null virus, suggesting that SM and ICP27 may regulate gene expression through a common pathway that is evolutionarily conserved between these two herpes viruses (Boyer, Swaminathan et al. 2002).

### Table 2.

<table>
<thead>
<tr>
<th>Herpes virus</th>
<th>Subfamily</th>
<th>ICP27 homologue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-herpes virus</td>
<td>varicella-zoster virus (VZV)</td>
<td>ORF4</td>
<td>(Inchauspe, Nagpal et al. 1989)</td>
</tr>
<tr>
<td></td>
<td>equine herpes virus type 1 (EHV-1)</td>
<td>UL3</td>
<td>(Zhao, Holden et al. 1992)</td>
</tr>
<tr>
<td>β-herpes virus</td>
<td>human cytomegalovirus (CMV)</td>
<td>UL69</td>
<td>(Chee, Bankier et al. 1990)</td>
</tr>
<tr>
<td>γ-herpes virus</td>
<td>Epstein-Barr virus (EBV)</td>
<td>SM</td>
<td>(Cook, Shanahan et al. 1994)</td>
</tr>
<tr>
<td></td>
<td>Herpes virus samiri (HVS)</td>
<td>ORF57</td>
<td>(Nicholas, Gompels et al. 1988)</td>
</tr>
<tr>
<td></td>
<td>human herpes virus 8 (HHV8-)/Kaposi's sarcoma associated virus (KSHV)</td>
<td>ORF57</td>
<td>(Bello, Davison et al. 1999)</td>
</tr>
</tbody>
</table>
7. Adenovirus

7.1 Introduction

Over the years, many important discoveries have been accomplished by the use of adenovirus as a model system. There are two genera of adenoviruses: Aviadenovirus (Avian) and Mastadenovirus (mammals). The adenoviruses that infect humans include more than 50 immunologically distinct types, subdivided into six subgenera, A-F (reviewed in (Shenk 1996; S.J Flint 2000)). Adenoviruses most commonly cause respiratory tract infections, like common cold, croup and bronchitis. Adenovirus has a linear double stranded DNA genome of about 36 kb, which is encapsulated into a non-enveloped icosahedral capsid particle.

Adenovirus enters the cell by endocytosis, thereafter the virus particle is partially dismantled, and the viral genome is deposited into the nucleus. The majority of the adenovirus genes are transcribed by the host cell RNA polymerase II (reviewed in (Cann 1993; Shenk 1996; S.J Flint 2000)). In cell cultures, transcription of the viral genome starts immediately upon entry into the nucleus and at 3-5 hours post-infection, approximately 15% of the mRNA in the cytoplasm is virus-encoded (reviewed in (Pombo 1995)). The adenovirus genes are classified into early and late genes. During the early phase, early region 1A (E1A) is the first gene to be transcribed. E1A encodes for transacting factors required for the expression of other early genes. The early phase of gene expression is followed by the onset of viral DNA replication, which in cultured cells occurs approximately 6-8 h after infection (reviewed in (Pombo 1995)). Replication of viral DNA is required for the transition to the late phase of gene expression. Almost all late genes are transcribed from the major late transcription unit, which by alternative splicing and alternative polyadenylation produces approximately 20 different mRNAs. Most of these mRNAs code for the structural components of the virion (reviewed in (Cann 1993; Shenk 1996; S.J Flint 2000)). Each cell can produce up to 10 000 new virus particles. By 32 h after infection, the cellular content of protein and DNA is about two fold higher than in normal cells (reviewed in (Pombo 1995)).
7.2 Nuclear organization in adenovirus infected cells

One of the first virus-induced structural changes observed in adenovirus-infected cells is small inclusions in the nucleus that rapidly increase in size as viral DNA synthesis continues. These inclusions are called viral replication centres and contain viral DNA and the viral 72K DNA binding protein (Puvion-Dutilleul and Pichard 1992). The number of replication centres is proportional to the multiplicity of the infection. Adenovirus DNA replication starts with a protein priming mechanism at either end of the viral genome. At each replication fork, only one of the two daughter strands is replicated producing a new duplex and a displaced single strand of DNA (ssDNA). In a second round of replication the displaced ssDNA serves as a template for second strand DNA synthesis (reviewed in (Cann 1993; Shenk 1996; S.J Flint 2000)). As the replication proceeds, the replication centres become larger and compartmentalized, with the single stranded DNA in the core of the centre surrounded by double stranded DNA that forms a network named the peripheral replication zone that is active in both replication and transcription (Puvion-Dutilleul and Pichard 1992; Pombo, Ferreira et al. 1994).

Intranuclear distribution of the splicing machinery varies during the course of an adenovirus infection (Bridge and Pettersson 1996). During the early stages of infection, shortly after the onset of viral replication, snRNPs and SR proteins are recruited to sites of viral transcription. They accumulate into ring-like structures surrounding the viral replication centres (Bridge, Carmo-Fonseca et al. 1993; Pombo, Ferreira et al. 1994; Bridge and Pettersson 1995; Bridge, Riedel et al. 1996). As the adenovirus infection proceeds and more late viral RNA is transcribed, splicing factors again accumulate in speckle-like structures that become progressively enlarged (Bridge, Riedel et al. 1996). These enlarged speckles concentrate splicing factors, poly(A) RNA and spliced viral mRNA (Bridge, Riedel et al. 1996), (Bridge, Xia et al. 1995). The localization of splicing factors in enlarged speckles has been shown to correlate with late mRNA export to the cytoplasm and production of late proteins (Aspegren, Rabino et al. 1998). This suggests that the enlarged speckles might have a posttranscriptional role in the production of RNA.
8. Present investigation

8.1 Project 1, papers I & II

8.1.1 Introduction
This study was initiated in order to investigate how the HSV-1 ICP27 protein induces an inhibition of pre-mRNA splicing and to increase our understanding of the basal mechanisms behind splicing. HSV-1 appears to attack essential splicing factor(s) and thereby potentially cause a complete shut-off of splicing. This is possible since the majority of HSV-1 genes are intron-less. Only four of the approximately 80 HSV-1 genes contain introns, and only one is expressed at late stages of infection. For this reason we believe that identification of the factor(s) that are targeted by ICP27 could be of general importance since it might reveal previously uncharacterized parts of the splicing machinery. Previously published in vivo and in vitro studies have been based on analyses of the splicing phenotype in HSV-1 wild type or ICP27 mutant virus infected cells. In this study, ICP27 was expressed in eukaryotic cells in the absence of other HSV-1 proteins.

8.1.2 Results and discussion
Paper I
With the development of an ICP27-expressing adenovirus vector, we were able to produce high amounts of the protein in eukaryotic cells in the absence of other HSV-1 proteins. The target cells are co-infected with a viral vector that express the trans-gene ICP27 and a trans-activator virus that constitutively produce an inducer-dependent trans-activator protein (Edholm, Molin et al. 2001). Pre-mRNA splicing was studied in a cell-free in vitro system, using two different 32P-labeled exogenous pre-mRNA substrates: the cellular β-globin pre-mRNA and the standard adenovirus derived pre-mRNA splicing substrate. The labeled exogenous pre-mRNA substrates were mixed with nuclear extracts and incubated under optimal conditions for in vitro splicing. The splicing activity in ICP27 containing nuclear extracts was compared with the activity in nuclear extracts prepared from cells infected with AdICP27 and the trans-activator virus, but without addition of the inducer. Combined results from these studies demonstrated that there is a drastic reduction of splicing products from both the cellular and the viral pre-mRNA transcript in ICP27-containing extracts. These results further suggest that the presence of ICP27 causes a general inhibition of splicing.
To determine at which stage of the splicing process the ICP27-mediated inhibition of splicing was manifested, we analyzed the formation of spliceosomal complexes. The results of this experiment showed a reduced rate of formation of the A-complex in ICP27-containing extract, indicating that the splicing inhibition is already manifested at or before the formation of the first energy-dependent spliceosomal A-complex. Further analysis of the conversion rates of the complexes, from A-complex to B-complex, and B-complex to C-complex showed that all complex conversion rates were reduced in ICP27-containing extracts. However, the largest difference between ICP27-containing extracts and control extracts appeared to be the formation of the mature spliceosomes, the C-complex. The different RNA-protein complexes formed in ICP27 extracts also migrated notably faster during electrophoresis than their counterparts formed in control extracts. This altered mobility might reflect a qualitative and/or a quantitative difference between the complexes formed in the two types of extracts. We also found that the splicing activity in the ICP27-containing extracts could be completely recovered by addition of small amounts of competent control extracts. This suggested that one or more essential splicing factors are post-transcriptionally modified in ICP27-containing extracts. When ICP27-containing extracts were added to the control extracts, inhibition of splicing was not transferred unless the extracts were preincubated at 30°C. Longer incubation times increased the level of splicing inhibition. These results could indicate that an enzymatic activity in the ICP27-containing extracts targets an essential component(s) of the splicing machinery.

To determine whether the splicing inhibition caused by ICP27 was specific, we compared RNA polymerase II activity in ICP27 extracts and control extracts. A DNA fragment containing the adenovirus major late promoter was incubated with 32P-labeled CTP in ICP27-containing extracts or control extracts under conditions optimal for RNA polymerase II transcription. The results demonstrated that there were no major differences in RNA polymerase II activity between the ICP27-containing extracts relative to the controls. This confirms that the splicing inhibition in ICP27 extracts is specific rather than a general toxic effect of an overexpressed protein.

Our observation that splicing is inhibited at an early stage of spliceosome formation, and that ICP27 causes a shift in mobility of the spliceosomal complexes is supported by another study performed by Bryant et al, in which they showed that ICP27 interacts with SAP145 during an HSV-1 infection (Bryant, Wadd et al. 2001). SAP145 is a component of the SF3b particle, which is implicated in tethering U2snRNP binding to the branch site and is therefore needed at an early stage of spliceosome assembly. Moreover, when ICP27 was
present, splicing *in vitro* was inhibited prior to the first catalytic step and B/C-complexes formed during splicing increased in mobility and reduced in intensity.

**Paper II**

With the conclusions drawn from paper I, our next goal was to identify cellular splicing factors that were targets for ICP27. We employed a biochemical complementation assay, in which whole cell extracts were fractionated using ammonium sulfate, and magnesium chloride precipitation steps. We found that several fractions from the ammonium sulfate precipitation could restore the splicing activity in splicing incompetent ICP27 extracts. The 90% ammonium sulfate precipitate, however, was most efficient in restoring the splicing activity. This fraction is highly enriched in the essential SR family of splicing factors. Since previous studies have shown that SR proteins are prime targets for viral control (Kanopka, Muhlemann et al. 1998; Estmer Nilsson, Petersen-Mahrt et al. 2001; Huang, Nilsson et al. 2002), we decided to continue with the 90% ammonium sulfate precipitate.

In the next step, we further purified the SR proteins present in the 90% ammonium sulfate fraction using magnesium chloride precipitation. The highly purified SR proteins were found to be very competent in activating splicing in ICP27-containing extracts. This suggest that SR proteins on their own are capable of reversing ICP27-mediated splicing inhibition. We also analyzed another essential splicing factor, U2AF65, for its capacity to restore splicing activity in ICP27-containing extracts. In this case, we did not observe any activation of the splicing activity, indicating that ICP27 specifically targets splicing factors of the SR protein family.

Our next question was whether individual SR proteins are capable of restoring splicing in ICP27 extracts. Also, it was important to exclude the possibility that the purified SR protein fraction contained components other than SR proteins that were responsible for the restoration of the splicing activity in ICP27-containing extracts. For this experiment we used a bacterially produced ASF/SF2 protein. ASF/SF2 was co-expressed in *E.coli* together with SR protein kinase 1, (SRPK1), which selectively phosphorylates serines in serine/arginine-rich regions. Addition of increasing amounts of phosphorylated ASF/SF2 activated splicing in ICP27-containing extracts. Collectively, these results suggest that ICP27 blocks splicing by inhibiting the SR protein family of splicing factors. Thus, addition of the whole family of SR proteins or a single SR protein is sufficient to reverse ICP27-mediated splicing inhibition.

The effects of SR proteins on splicing depend upon the phosphorylation status of the protein. SR proteins in their active form are phosphorylated, whereas both
hypophosphorylation and hyperphosphorylation decrease their activity as splicing factors (Kanopka, Muhlemann et al. 1998), (Xiao and Manley 1997). To investigate whether ICP27 inhibits splicing by changing the phosphorylation status of SR proteins, we analyzed different ICP27-containing nuclear extracts. We found that the majority of the SR proteins were dephosphorylated in extracts containing ICP27. Finally, we analysed whether phosphorylation of SR proteins in the ICP27-containing extracts could restore the splicing activity. For this experiment we added purified SRPK1 to ICP27 extracts. Although the effect was less dramatic compared to addition of ASF/SF2, or purified SR proteins, addition of SRPK1 also resulted in an increase in the splicing activity in ICP27 extracts.

The inactivation of SR proteins by ICP27-mediated dephosphorylation is thus perhaps reversible, since addition of a kinase to the splicing reaction probably phosphorylates SR proteins to the extent that they can activate splicing. Another indication that SR proteins are reversibly dephosphorylated in ICP27-containing extracts comes from the observation that addition of SR proteins purified from ICP27-expressing cells restores splicing activity to ICP27 extracts. Western blot and coomassie analysis of the proteins in the ICP27 SR fraction demonstrated that they were phosphorylated. It is most likely the SR proteins we purify from the whole cell extract become phosphorylated during the purification process and regain their activity as splicing factors.

Phosphorylation of SR proteins also influences their sub cellular distribution: dephosphorylated SR proteins are localized to nuclear speckles whereas phosphorylated SR proteins are localized to sites of active transcription (Koizumi, Okamoto et al. 1999). Previous studies have shown that essential splicing factors are redistributed in HSV-infected cells (Phelan, Carmo-Fonseca et al. 1993; Sandri-Goldin, Hibbard et al. 1995). Since SR proteins in nuclear extracts prepared from ICP27-expressing cells were found to be dephosphorylated, we analysed the distribution of ASF/SF2 in ICP27-expressing cells by immunofluorescence. HeLa cells were transfected with a plasmid expressing GFP-ICP27 and the distribution of endogenous ASF/SF2 was monitored by monoclonal antibody 103. In cells expressing ICP27, ASF/SF2 accumulated in speckles, which are noticeable larger than in control cells. Moreover, ICP27 is not present in the enlarged speckles with ASF/SF2, and instead, often appears to be surrounding them. This result showed that expression of ICP27 causes a redistribution of ASF/SF2 in the nucleus, and that this may be a consequence of an ICP27-mediated dephosphorylation of ASF/SF2.
Based on these results, we concluded that the SR protein family of splicing factors are main targets for ICP27 and that the activity of SR proteins as regulatory splicing factors is reduced in ICP27-expressing cells by dephosphorylation.

A major finding was recently published by Sciabica et al (Sciabica, Dai et al. 2003). In this study they show that ICP27 interacts with SRPK1 to mediate splicing inhibition by altering the SR protein phosphorylation. Sciabica et al also reported that during an HSV-1 infection, phosphorylation of several SR proteins was reduced and that this correlated with a sub-nuclear redistribution of the SR proteins. Further, ICP27 was shown to interact specifically with SRp20 both in vivo and in vitro. ICP27 interaction with SRPK1, alters SRPK1’s activity and relocates it to the nucleus. In conclusion, they propose that ICP27 recruits SRPK1 to the nucleus and interacts with SR proteins at the site of spliceosome assembly. Their finding thus supports our observation that SR proteins are dephosphorylated in ICP27-expressing cells.

8.1.3 Perspectives
To date, two other DNA viruses, adenovirus and vaccinia virus have been reported to regulate the cellular splicing machinery by inducing a dephosphorylation of the SR family of splicing factors (Kanopka, Muhlemann et al. 1996; Estmer Nilsson, Petersen-Mahrt et al. 2001; Huang, Nilsson et al. 2002). SR proteins prepared from late adenovirus-infected cells are inactivated by a virus-induced partial de-phosphorylation. This modification enhances the production of the alternatively spliced late mRNA IIIa, which otherwise is repressed by phosphorylated SR proteins binding to an intronic repressor element, the 3RE (Kanopka, Muhlemann et al. 1998). Presumably, splicing of most late stage-specific adenovirus mRNAs is stimulated by this virus-induced dephosphorylation of SR proteins. Most adenovirus late genes are expressed from the major late transcription unit, which by alternative splicing and alternative polyadenylation, produce approximately 20 different mRNAs. Adenovirus is therefore dependent on the cellular splicing machinery for processing its own pre-mRNAs. During an adenovirus infection, the splicing machinery is not functionally blocked, and instead, a partial dephosphorylation of SR proteins appears to promote a temporal shift into the late stage of infection and production of late viral mRNAs.

Vaccinia virus, on the other hand, replicates in the cytoplasm and encodes for genes lacking introns. This suggests that this virus could benefit from a total block of the splicing machinery for maximal expression of its own genes. When SR proteins from vaccinia virus-infected cells were analysed, they were indeed found to be hypophosphorylated and
functionally inactivated as splicing regulatory proteins. Re-phosphorylation of these SR proteins only partially restored the splicing enhancer or splicing repressor activity (Huang, Nilsson et al. 2002). It is possible that vaccinia viruses use an alternative, or additional mechanism, to alter the phosphorylation status of the SR proteins compared to adenovirus. Adenovirus has been shown to induce dephosphorylation of SR proteins by the virus-encoded E4ORF4, which binds to the cellular protein phosphatase 2A, (PP2A), and directs it to SR proteins for dephosphorylation. So far, it has not been elucidated how vaccinia virus inactivates SR proteins as splicing regulatory proteins. However, vaccinia virus encode for its own dual protein phosphatase, VH1, which is required for virus growth and gene expression (Liu, Lemon et al. 1995). VH1 may be a possible candidate for causing SR protein dephosphorylation.

In this study, we present a third DNA virus, HSV-1, which uses the SR protein family of splicing factors to regulate the splicing machinery. HSV-1 only has four intron-containing genes, so it would benefit from blocking most of the splicing activity in the cell. In the study presented in paper II, the SR family of splicing factors appeared to be functionally inactivated by a reversible dephosphorylation. One question that remains is how the SR proteins are dephosphorylated and whether ICP27 targets more components of the splicing machinery to induce splicing inhibition. The accumulation of hypophosphorylated SR proteins in HSV-1-infected cells could be induced by at least three different mechanisms; i, by inhibiting a protein kinase(s) responsible for SR protein phosphorylation; ii, by inducing dephosphorylation by a phosphatase and iii, by inducing or encoding for a protein that blocks SR protein phosphorylation.

Sciabica et al have proposed that ICP27 prevents SRPK1 phosphorylation of SR proteins by interacting with and changing its activity and cellular localization (Sciabica, Dai et al. 2003). ICP27 has been shown to interact with numerous proteins (listed in Table 1, page 25). The cellular p32 is another protein with which ICP27 interacts, and which could play a role in the ICP27 induced inhibition of splicing (Bryant, Matthews et al. 2000). p32 has been shown to interact with and inhibit ASF/SF2 as a splicing factor by blocking phosphorylation of its RS domain (Petersen-Mahrt, Estmer et al. 1999). It is possible that the interaction of ICP27 with p32 is used to direct p32 to SR proteins to inhibit their phosphorylation.
8.2 Project 2, paper III

8.2.1 Introduction
This study was undertaken in order to investigate how cellular splicing factors are recruited to sites of splicing in the cell nucleus. We compared the recruitment of SR-proteins ASF/SF2 and SC35 to active sites of splicing with splicing factors U2AF$^{65}$ and snRNPs, during an adenovirus infection. We also analysed the contribution of the different domains of ASF/SF2 for the recruitment to active sites of viral transcription and splicing.

8.2.2 Results and discussion
In order to analyse the spatial distribution of ASF/SF2 during an adenovirus infection, HeLa cells were infected with adenovirus and fixed and permeabilized after different times of infection. The sub-nuclear distribution of ASF/SF2 was first determined by immunofluorescence confocal microscopy using a monoclonal antibody directed against the endogenous protein. The viral replication centres were visualized using a separate antibody directed against the viral 72K DNA-binding protein. The results showed that the distribution of ASF/SF2 during an adenovirus infection is dramatically altered. At an intermediate stage of infection, at 12-16 hours post-infection (hpi), almost all detectable ASF/SF2 had accumulated at sites surrounding the viral replication centres. It is in the immediate vicinity of these structures that transcription and splicing is predominantly detected. This indicates that ASF/SF2 is recruited to the nuclear sites where adenovirus genes are transcribed and processed. At late stages of infection, 20 hpi, ASF/SF2 is found in so-called enlarged speckles distant from the replication centres.

The staining pattern from endogenous ASF/SF2 detected with the monoclonal antibody was confirmed by transient expression of a myc- or Green Fluorescent Protein-tagged (GFP) version of the protein. The transfected cells were subsequently superinfected with adenovirus. Both the myc- and the GFP-tagged ASF/SF2 proteins displayed similar, if not identical, distribution pattern to endogenous ASF/SF2 during an adenovirus infection. U2AF$^{65}$ is similar to ASF/SF2 in that it contains an RNA-binding domain and an RS domain. Previous studies have demonstrated that an adenovirus infection induces a redistribution of U2AF$^{65}$ into ring-like structures that colocalize with the sites of viral transcription (Gama-Carvalho, Krauss et al. 1997). GFP-ASF/SF2 distribution was compared with U2AF$^{65}$ in order to examine if ASF/SF2 is recruited to the same ring-like structures as U2AF$^{65}$ during an
adenovirus infection. Both proteins were present in ring-like structures at the intermediate stage of infection and in enlarged speckles at the late phase of infection. When superimposing the GFP-ASF/SF2 and U2AF65 signals, it is apparent that they colocalize during the adenovirus infection. Although U2AF65 appears to be more evenly distributed throughout the nucleus of an infected cell compared to ASF/SF2, they show a clear colocalization in the ring-like structures. SC35 and ASF/SF2 are similar in size and both play a role in constitutive and alternative splicing. However, SC35 has only one RRM and it does not shuttle between the cytoplasm and the nucleus. When ASF/SF2 distribution is compared to that of SC35 during an adenovirus infection, they display the same pattern of redistribution. Further, a confocal overlay of the signals shows an almost identical distribution pattern. This demonstrates that SC35, like ASF/SF2, accumulates in the ring-like structures in which adenovirus genes are actively transcribed and processed.

Experiments with mutant proteins showed that the two RNA-binding domains present in ASF/SF2 are sufficient for efficient recruitment of the protein to the ring-like structures surrounding viral replication centres. The ASF mutant, in which the two RNA binding domains were deleted (GFP-RS), was evenly distributed throughout the nucleus and was not notably redistributed during an adenovirus infection. The protein did not accumulate in ring-like structures or enlarged speckles during the infection.

These results indicate that ASF/SF2 is recruited to sites of viral transcription and splicing by a different mechanism compared to that previously shown for the essential splicing factor U2AF65 (Gama-Carvalho, Krauss et al. 1997). Localization of U2AF65 to these sites is totally dependent upon an intact RS domain. The RS domains of both ASF/SF2 and U2AF65 have been shown to mediate protein-protein interactions with other splicing factors. In the case of U2AF65, the RS domain has also been shown to be necessary for efficient binding of U2 snRNA to the branch site in pre-mRNA, perhaps by binding to one of the two RNA components (Valcarcel, Gaur et al. 1996). Previous studies have shown that a virus-induced dephosphorylation of the majority of the SR proteins, including ASF/SF2, late during an adenovirus infection reduces their activity as splicing enhancer or splicing repressor proteins (Kanopka, Muhlemann et al. 1998). Despite this, we observed that ASF/SF2 accumulates in ring-like structures where late adenoviral genes are transcribed and their pre-mRNAs processed. This may suggest that the hypophosphorylated SR proteins are also needed for late viral mRNA processing or that the SR proteins are required for some process other than splicing. ASF/SF2 has been shown to interact directly both with the general mRNA export factor TAP (Huang, Gattoni et al. 2003) and with the transcriptional coactivator, p52...
(Ge, Si et al. 1998). Thus, it is possible that ASF/SF2 may be required for efficient transcription of adenovirus genes and/or in export of viral mRNAs.

8.2.3 Perspectives
Cellular compartments are important for functions of the cell and for the regulation of gene expression (reviewed in (Carmo-Fonseca 2002)). Molecules can move between cellular compartments in different ways. Nuclear compartments differ from most cytoplasmic compartments in that they are not surrounded by membranes. Recent data from imaging of the motion of macromolecules inside the living cells have shown that splicing factors are in continuous flux between nuclear compartments which are not separated by a physical barriers (Eils, Gerlich et al. 2000). As proteins diffuse through the nuclear space, they transiently accumulate in a steady-state compartment by interactions with high-affinity binding sites. According to this view, sequences and domains that are necessary for proper localization within the cell, for example the nuclear retention signal within hnRNP C, would more likely act as retention signals rather than as targeting or transport signals. Consequently, the recruitment of splicing factors to sites of splicing would not be a directed process where the factors are transported from storage in speckles to the active splice site. Instead, the steady-state level of the factors would increase dynamically at the active splice site because of the prolonged binding and interactions by the factors. The RS domain of ASF/SF2 has been reported to function in the dissociation of the protein from speckles, and phosphorylation of serine residues is a prerequisite for this event (Misteli, Caceres et al. 1998). It is possible that the dephosphorylated RS domain has a higher binding affinity to the other components of the speckles, which cause retention of the protein in speckles. The observation from this study that the RS domain of ASF/SF2 is not needed for accumulation of the protein at sites of viral transcription and splicing implies that interactions with the RS domain are not sufficient to anchor the protein at the transcription/splice site. Instead it is the affinity of the RRM2s of ASF/SF2 that anchor the protein to the site of active splicing. Whether ASF/SF2 is anchored to the activated viral gene by interaction with an RNA component and/or a protein and whether this is unique for viral genes or is universal for metazoan genes remains to be established.
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10. References


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