A single RNA recognition motif in splicing factor ASF/SF2 directs it to nuclear sites of adenovirus transcription

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SR protein ASF/SF2 is a general pre-mRNA splicing factor as well as a regulator of alternative splicing. Data presented here show that ASF/SF2 is efficiently recruited to sites in the nucleus where adenovirus genes are transcribed and the resulting pre-mRNAs are processed. At the intermediate stages of a productive infection, ASF/SF2 colocalizes with small nuclear ribonucleoprotein particles (snRNPs), splicing factors in ring-like structures surrounding viral replication centres and, at late stages of the infection, in enlarged speckles. Results presented here demonstrate that ASF/SF2 requires only one of the two RNA-recognition motifs (RRMs) present in the protein for its efficient recruitment to the ring-like structures, where viral pre-mRNAs are transcribed and processed, and that the arginine/serine-rich (RS) domain in ASF/SF2 is both redundant and insufficient for the translocation of the protein to active viral RNA polymerase II genes in adenovirus-infected cells.

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Human adenovirus type 5 (family Adenoviridae, genus Mastadenovirus) is a non-enveloped icosahedral virus containing a linear double-stranded DNA molecule that replicates in the cell nucleus. In a productive adenovirus infection there is a dramatic reorganization of the cell nucleus during the intermediate and late stages of lytic infection. At intermediate stages, essential pre-mRNA splicing factors, e.g. snRNPs, which are normally evenly distributed over the entire nucleoplasm, and concentrated in so-called splicing speckles, become concentrated in ring-like structures surrounding viral replication centres (also referred to as peripheral replicative zones) (Bridge et al., 1993, 1995). Virus-induced ring-like structures represent nuclear sites where newly replicated adenovirus DNA is heavily transcribed by RNA Pol II and where the resulting pre-mRNAs are processed (Pombo et al., 1994). At the late stages of lytic infection, splicing factors are further redistributed into sites that resemble enlarged speckles, situated relatively distant from viral replication centres. This type of structure in adenovirus-infected cells has been shown to contain snRNPs and spliced adenovirus mRNAs (Bridge et al., 1996).

Immunolocalization studies of adenovirus-infected cells have shown that the SR protein family of splicing factors is redistributed as a consequence of adenovirus infection. Although these studies clearly indicated that the vast majority of SR proteins are dramatically relocalized in adenovirus-infected cells, they did not address how individual SR proteins are affected (Bridge et al., 1995). The present study was initiated in order to study how SR protein ASF/SF2 is distributed during the course of a lytic adenovirus infection in cultured cells. SR proteins are essential splicing factors and regulators of alternative pre-mRNA splicing (reviewed by Graveley, 2000). All SR proteins have a modular organization containing an N-terminal RNA-binding domain consisting of either one or two RRMs, and a C-terminal domain RS domain containing repetitive arginine–serine dipeptides that mediates protein–protein interactions with components of the splicing machinery. In this study, we also investigated which functional domains of ASF/SF2 are required for its accumulation at sites of virus transcription and splicing.

In order to analyse the spatial distribution of SR protein ASF/SF2 during a lytic adenovirus infection, HeLa cells were infected with wild-type adenovirus (dl309) and at various time-points the cells were fixed and permeabilized (Gama-Carvalho et al., 1997). The subnuclear distribution of ASF/SF2 was examined using immunofluorescence confocal microscopy. The distribution of endogenous ASF/SF2 (detected with mAb 103; Hanamura et al., 1998) in infected cells was compared to that of adenovirus 72 kDa DNA-binding protein (72K DBP) (detected with anti-72K; Linne et al., 1977). The structures visualized by 72K DBP antibodies have been referred to as virus-induced compact
ring structures and, besides 72K DBP, they contain newly replicated ssDNA. Virus DNA replication occurs at the surface of compact ring structures and transcription and splicing are predominantly detected around these structures (Pombo et al., 1994).

In uninfected HeLa cells, mAb 103 displayed a dispersed staining throughout the nucleoplasm with additional concentrations in speckles (Fig. 1a and Caceres et al., 1998). The distribution of ASF/SF2 was dramatically altered during an adenovirus infection (Fig. 1A, b and c). At an intermediate stage of infection [12–16 h post-infection (p.i.)] ASF/SF2 was predominantly detected in ring-like structures, at sites close to, and surrounding, viral replication centres (Fig. 1A, b). In Fig. 1(A, c) the most common mAb 103 staining found in cells is shown, where the infection cycle had entered the late stage (16–20 h p.i.). In these cells, ASF/SF2 accumulates in structures that resemble enlarged speckles.

In order to confirm the distribution pattern for ASF/SF2 in adenovirus-infected cells using antibody mAb 103, a tagged version of ASF/SF2 was transiently expressed in cells subsequently superinfected with adenovirus [ASF/SF2 was fused in-frame with GFP-coding sequence in the eukaryotic expression vector pEGFP-C1 (Clontech)]. Cells infected with adenovirus were transfected 5–6 h before infection.

As previously reported by others (Misteli et al., 1997; Sleeman et al., 1998), a GFP-tag does not appear to significantly alter ASF/SF2 distribution in uninfected cells (compare Fig. 1A, a and d). Furthermore, the distribution of GFP–ASF/SF2 throughout the intermediate and late stages of adenovirus infection strongly resembles what was observed for endogenous ASF/SF2 with mAb 103 (Fig. 1A, d, e). The same result was observed when a Myc-tagged version of ASF/SF2 was analysed (data not shown). These results combined show that ASF/SF2 is redistributed in the nuclei of adenovirus-infected cells and that endogenous and tagged ASF/SF2 are similarly, if not identically, distributed in non-infected and in adenovirus-infected cells.

ASF/SF2 contains three characterized structural domains: two RRMs and one RS domain (Caceres & Krainer, 1993; Zuo & Manley, 1993). In order to determine the role of the different domains in recruitment of ASF/SF2 to nuclear splicing sites in adenovirus-infected cells, the distribution of transiently expressed mutant derivatives of the protein were analysed. Two main types of ASF/SF2 mutants were studied: protein derivatives lacking the entire RS domain (ΔRSb; contains amino acids 1–183) or ASF/SF2 mutants in which one or both RRMs had been deleted or crippled (RRM1/RS, amino acids 112–197 are deleted; RRM2/RS, RRM1 is crippled by point mutations F56D and F58D; RS181, contains amino acids 181–248). Plasmid DNAs encoding the different ASF/SF2 derivatives were transfected into HeLa cells that were subsequently mock infected or infected with adenovirus. Expression of all tagged ASF/SF2 mutants was verified by SDS-PAGE and Western-blotting analysis using appropriate antibodies (data not shown). The distribution of ASF/SF2 proteins and viral replication centres were determined as previously described.

In uninfected HeLa cells, the ASF/SF2 mutant that lacks the entire C-terminal RS domain, ΔRSb, displayed a diffuse nuclear distribution with noticeable accumulation in speckled structures, although not to the same extent as the wild-type (Fig. 1B, a). A striking difference between the localization of ΔRSb and wild-type ASF/SF2 was that the mutant protein was not excluded from nucleoli (Fig. 1B, a). More importantly, localization of ΔRSb in cells infected by adenovirus was almost indistinguishable from wild-type ASF/SF2. At an intermediate phase of adenovirus infection, large amounts of ΔRSb accumulated in ring-like structures surrounding viral replication centres (Fig. 1B, b), and, at later stages of the infection ΔRSb, accumulated in virus-induced enlarged speckles (data not shown).

The distribution of ASF/SF2 mutants RRM1/RS and RRM2/RS (Fig. 1B, c and e) was analysed next. In uninfected cells, both the RRM1/RS protein and the RRM2/RS protein were found to be almost exclusively nuclear, where they displayed a nucleoplasmic and speckled distribution pattern resembling the wild-type protein and ΔRSb mutant, but, unlike ΔRSb, they were largely excluded from nucleoli. Importantly, both the RRM1/RS protein and the RRM2/RS protein were found to be efficiently recruited into ring structures in adenovirus-infected cells (Fig. 1B, d and f). At later stages of the virus life cycle, they both accumulated in a few enlarged speckles (data not shown).

The role of the RS domain in subnuclear localization of ASF/SF2 was determined next. Although RS181 was

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Fig. 1. (A) Adenovirus infection induces redistribution of ASF/SF2 in the nucleus. HeLa cells were either mock infected (a, d) or infected with adenovirus (b, c, e and f), before they were fixed and permeabilized. Double labelling of cells with mAb 103 (green) and anti-72K against the viral DNA-binding protein (red) is shown in a–c. The position of a viral replication centre surrounded by ASF/SF2 is indicated by an arrow in 1b. In 1c, the arrow indicates the position of an enlarged speckle. In d–f HeLa cells were transiently transfected with GFP–ASF/SF2 and thereafter infected with adenovirus. Cells transfected with GFP–ASF/SF2 were labelled with anti-72K (red) (d–f). (B) Recruitment of ASF/SF2 to splicing sites is independent of the RS domain. HeLa cells were transiently transfected with ASF/SF2 mutants ΔRSb, RRM1/RS, RRM2/RS or RS181. The cells were then either mock infected (a, c, e and g) or infected with adenovirus (b, d, f and h). The viral DNA-binding protein was labelled with anti-72K (red), and ASF/SF2 mutant signals are shown in green. (C) Endogenous ASF/SF2 is recruited to ring-like structures in ASF/SF2 mutant RS181-expressing cells. HeLa cells were transfected with RS181 (green) and infected with adenovirus. At 12 h.p.i. the cells were fixed and labelled with mAb 103 detecting endogenous ASF/SF2 (red).
detected throughout the cell, the vast majority of the protein appeared to accumulate in the nucleus (Fig. 1B, g). RS181 was dispersed throughout the nucleoplasm with some enrichment in speckles. Similar to ΔRSb, RS181 was not excluded from the nucleolus. Importantly, RS181 was not obviously redistributed upon adenovirus infection.

### Fig. 2.
Wild-type ASF/SF2 (a–c) and the ASF/SF2 mutants ΔRSb (d–f), RRM1/RS (g–l), RRM2/RS colocalize with snRNPs in ring-like structures at intermediate stages of infection. HeLa cells transfected with wild-type ASF/SF2 or with ASF/SF2 mutants (a, d, g, j, m) (green) were infected with adenovirus and subsequently immunolabelled with anti-SM, C45 antibody detecting snRNPs (b, e, h, k, n) (red).
(Fig. 1B, h). Although RS181 was present in close proximity to viral replication centres, the protein did not accumulate in virus-induced ring-like structures at intermediate stages of the infection. Instead, the vast majority of RS181 remained diffusely distributed throughout the nucleus, even at stages of the lytic life cycle when most of wild-type ASF/SF2 accumulates in enlarged speckles. RS181 was even detected within the viral replication centres from which wild-type ASF/SF2 and RS deletion mutants normally are excluded (e.g. compare Fig. 1A, b and IB, h).

The expression of RS181 did not alter the distribution of endogenous ASF/SF2 in uninfected (data not shown) or in infected cells. Fig. 1C shows a cell at the intermediate stage of infection, where ASF/SF2 is detected in ring-like structures and RS181 is dispersed over the nucleoplasm and also is present in the centres of the ring-like structures. These results show that RS181 protein does not prevent ASF/SF2 from being recruited to sites of mRNA processing.

Earlier work has shown that, in adenovirus-infected cells, several essential splicing factors are recruited to ring-like structures that resemble the ones observed for ASF/SF2 and the mutants ΔR5b, RRM1/RS and RRM2/RS above, and that these structures constitute nuclear sites where adenovirus genes and pre-mRNAs are transcribed and processed. In order to examine whether the ring-like structures, in which ASF/SF2 and the mutants ΔR5b, RRM1/RS and RRM2/RS accumulate, are identical to such structures, the distribution of these proteins was compared to snRNPs in a double-labelling experiment (snRNPs were detected with c45; Gama-Carvalho et al. 1997). The wild-type ASF/SF2 and the mutants ΔR5b, RRM1/RS and RRM2/RS were found to largely colocalize with snRNPs in speckles in uninfected cells (data not shown). In infected cells, they colocalized in ring-structures at intermediate stages of infection (Fig. 2, a–l) and in enlarged speckles at late stages of infection (data not shown). These data confirm that the ring-like structures that ASF/SF2 and the mutants, except RS181, accumulate in, are truly nuclear sites where adenovirus mRNAs are processed. Also, although RS181 was not recruited to sites where adenovirus genes are transcribed, snRNPs had accumulated in ring-like structures (Fig. 2, m–o). The RS181 results, and those obtained with ASF/SF2 mutant derivatives ΔR5b, RRM1/RS and RRM2/RS, demonstrate that recruitment of ASF/SF2 to nuclear sites of adenovirus transcription and splicing is largely determined by RRM2 and that the presence of an RS domain is neither sufficient, nor necessary, for this to happen.

To the best of our knowledge, the only known functional domains left in the ASF/SF2 mutant ΔR5b are the two RRM2, which implies that ASF/SF2 is recruited to virus transcription sites mainly by binding to a RNA component. In support of this, in adenovirus-infected cells U2AF65, recruitment to nuclear sites of virus splicing is totally dependent upon an intact RS domain (Gama-Carvalho et al., 1997). This domain in U2AF65 has been found to be necessary for efficient binding of U2 snRNA to the branch site in the pre-mRNA, perhaps by direct interaction with one of the two RNA components (Valcarcel et al., 1996). Furthermore precedence exists, since localization of the polypyrimidine tract binding protein-associated splicing factor (PSF) is dependent upon its second RRM (Dye & Patton, 2001). Thus, in at least two cases, the RRMs largely determine the intranuclear distribution of proteins. However, it should be noted that we have found that mRNA-binding capacity, as such, probably is not sufficient for a protein to be recruited to the virus-induced ring-like structures, since two nuclear mRNA-binding proteins, hnRNP A1 [hnRNP A1 was fused in-frame with GFP-coding sequence in the eukaryotic expression vector pEFGP-C1 (Clontech)] and PSP1 (YFP–PSP1, Fox et al., 2002), do not accumulate in ring-like structures where adenovirus genes are transcribed (Fig. 3).

The fact that recruitment of ASF/SF2 to sites of virus splicing is exclusively dependent on its RRM2, and that the RS domain appears to be redundant for this, opens up questions on how ASF/SF2 recruitment to these sites occurs. Studies of ASF/SF2 dynamics in living cells have shown that it rapidly moves between the nucleoplasm and speckles, in and out from various compartments perhaps in search of appropriate binding partners. It is possible that activated viral genes, at the surface of viral replication centres, trap ASF/SF2 and other roaming splicing factors by binding to the RRM2. Whether the trap which occurs at activated viral genes is an RNA component and/or a protein, and whether this is unique for viral genes or universal for metazoan genes, remains to be established.

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