



The fate of U1 snRNP during anti-Fas induced apoptosis: specific cleavage of the U1 snRNA molecule

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Abstract

During apoptosis, the U1-70K protein, a component of the spliceosomal U1 snRNP complex, is specifically cleaved by the enzyme caspase-3, converting it into a C-terminally truncated 40-kDa fragment. In this study, we show that the 40-kDa U1-70K fragment is still associated with the complete U1 snRNP complex, and that no obvious modifications occur with the U1 snRNP associated proteins U1A, U1C and Sm-B/B'. Furthermore, it is described for the first time that the U1 snRNA molecule, which is the backbone of the U1 snRNP complex, is modified during apoptosis by the specific removal of the first 5–6 nucleotides including the 2,2,7-trimethylguanosine (TMG) cap. The observations that U1 snRNA cleavage is very specific (no such modifications were detected for the other U snRNAs tested) and that U1 snRNA cleavage is markedly inhibited in the presence of caspase inhibitors, indicate that an apoptotically activated ribonuclease is responsible for the specific modification of U1 snRNA during apoptosis. *Cell Death and Differentiation* (2000) 7, 70–79.

Keywords: U1 snRNP; U1-70K; U1 snRNA; apoptosis; caspase

Abbreviations: Ac, acetyl; CMK, chloromethylketone; DMSO, dimethyl sulfoxide; DTE, dithioerythritol; FasL, Fas-Ligand; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FMK, fluoromethylketone; mAb, monoclonal antibody; NP-40, nonidet P-40; nt, nucleotide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PI, propidium iodide; Ψ, pseudouridine; scFv, single chain variable fragment; SDS, sodium dodecyl sulfate; SLE, systemic lupus erythematosus; snRNP, small nuclear ribonucleoprotein; SR protein/domain, serine-arginine rich protein/domain; TMG, 2,2,7-trimethylguanosine; Z, benzyloxycarbonyl

Introduction

The U1 small nuclear ribonucleoprotein (U1 snRNP) complex is a common target for autoantibodies present in the serum of patients with systemic lupus erythematosus (SLE) and SLE-overlap syndromes.^{1,2} The U1 snRNP complex contains the U1 snRNA molecule and the U1 snRNP specific proteins U1-70K, U1A, and U1C plus a common set of eight proteins, called the Sm proteins. Most of these individual components, including the U1 snRNA,^{3–5} have been shown to be a target of patient autoantibodies (reviewed by Klein Gunnewiek *et al.*)⁶

The mechanisms by which these components of the U1 snRNP complex and other autoantigens escape immunological tolerance are largely unknown, although a number of recent observations suggest that modified self-proteins that are generated during apoptosis (i.e. programmed cell death)⁷ or necrosis,⁸ may play an important role in the development of autoimmunity. It has been shown that autoantigens targeted in SLE become concentrated in two discrete populations of surface structures on apoptotic cells, namely apoptotic bodies and apoptotic surface blebs.^{9–11} At present, the apoptotic cleavage of many of these autoantigens by caspases, a family of cysteinyl aspartate-specific proteinases,¹² has been documented (reviewed in Utz and Anderson).¹³ Since most of these autoantigens have important functions in the cell, their cleavage during early stages of apoptosis may be expected to silence basic cellular processes.

For example, the U1 snRNP complex is involved in pre-mRNA splicing¹⁴ and the U1-70K protein plays an essential role in this process.¹⁵ During apoptosis, the U1-70K protein is specifically cleaved by the enzyme caspase-3, converting it into a C-terminally truncated 40-kDa protein.^{16,17} It is possible that the loss of the C-terminus, which contains an SR-domain known to be important for its interaction with other splicing factors,⁶ renders the U1-70K protein non-functional, thus abrogating the process of mRNA splicing. It is currently unknown whether this 40-kDa cleavage product is still associated with the U1 snRNP complex in apoptotic cells.

In addition to caspase cleavage, other modifications of autoantigens have been described. It has been shown recently that at least four hyperphosphorylated SR proteins, i.e. pp54, pp42, pp34, and pp23, become associated with the apoptotic U1 snRNP complex.¹⁸ As discussed in detail elsewhere, the altered localization of the U1 snRNP complex in apoptotic cells, together with modifications of the complex during cell death (i.e. caspase-mediated cleavage of U1-70K and the association with hyperphosphorylated SR proteins) may be critical determinants in the development of an immune response to the U1 snRNP complex in susceptible individuals.¹³ In this context it

should be noted that the U1 snRNA molecule is also a major target of autoimmunity in SLE-overlap syndromes,^{1,2} and that changes in the titer of anti-U1 snRNA auto-antibodies appear to correlate with the severity of the disease.⁵ Therefore, it would be interesting to investigate whether the U1 snRNA molecule is also modified during apoptosis.

In this study, we have analyzed the fate of the U1 snRNP complex during Fas-induced apoptosis of Jurkat cells. We show that the U1 snRNA molecule is modified by the specific loss of the first 5–6 nucleotides including the 2,2,7-trimethylguanosine-cap (TMG) during anti-Fas induced apoptosis. Furthermore, it is shown that the 40-kDa apoptotic fragment of the U1-70K protein remains associated with the U1 snRNP complex, and that the U1A, U1C and Sm-B/B' proteins remain associated as well, but are not detectably modified.

Results

The extent of anti-Fas (mAb 7C11) induced apoptosis in Jurkat cells was assessed by flow cytometry using annexin V-FITC and propidium iodide (Figure 1). Annexin V stains phosphatidylserine, a marker for the early apoptotic state,¹⁹ whereas staining with propidium iodide is indicative for a late state of apoptosis (secondary necrosis). Using annexin V staining it was established that 57.1% of the Jurkat/control cells were apoptotic 2 h after addition of the anti-Fas antibody, while this percentage increased to 79.3% after 4 h, and 90.3% after 8 h. At the latter time point only 9.1% of the Jurkat/control cells were permeable to propidium iodide.

The 40-kDa protein fragment of U1-70K remains associated with the U1 snRNP complex in apoptotic cells

During apoptosis, the U1-70K protein is specifically cleaved by the enzyme caspase-3, converting it into a C-terminally truncated 40-kDa protein.^{16,17} For the detection of both U1-70K and the 40-kDa protein fragment in non-apoptotic and apoptotic cell lysates we routinely used an SLE patient serum (i.e. H42) containing polyclonal antibodies directed to the U1-70K protein. The characteristic apoptotic 40-kDa cleavage

product was already detectable 2 h after anti-Fas mAb addition in Jurkat/control cells, and gradually increased to a maximum level at 8 h. At the latter time point hardly any uncleaved U1-70K was detectable (Figure 2A, left panel). In Jurkat/Bcl-2 cells, in which the apoptotic process is inhibited,^{20,21} only a small amount of U1-70K was cleaved (Figure 2A, right panel).

To investigate whether the 40-kDa cleavage product remains associated with the U1 snRNP complex in apoptotic cells, we immunoprecipitated U1 snRNP complexes derived from non-apoptotic and apoptotic cells using monoclonal antibodies directed to the U1 snRNP specific proteins U1A (mAb 9A9, which also recognizes U2B')²² and U1C (scFv K36),²³ and to the common or Sm-proteins Sm-D1 (mAb KSm2)²⁴ and Sm-B/B' (mAb KSm5).²⁴ Immunoprecipitates were then analyzed by immunoblotting using the anti-U1-70K serum H42 (Figure 2B). In all cases the 40-kDa cleavage product was found in the immunoprecipitate of apoptotic Jurkat/control cell lysates indicating that the 40-kDa protein fragment (as well as the U1A, U1C, Sm-D1 and Sm-B/B' proteins) is still associated with the U1 snRNP complex in apoptotic cells. Note that probably due to antibody titer differences the efficiencies of immunoprecipitations by the various antibodies showed some variation. Interestingly, the data obtained with the very efficiently precipitating anti-Sm-B/B' antibody indicate that there is essentially no release of the 40-kDa fragment from the complex (Figure 2B, lanes 13–16). Electrophoretic separation in two dimensions of immunoprecipitated apoptotic U1 snRNP complexes confirmed that the 40-kDa cleavage product and phosphorylated SR proteins are associated with the U1 snRNP complex during Fas-induced apoptosis (¹⁸ and results not shown).

As reported before by Casiano *et al.*²⁵ we also found that the U1A, U1C, and Sm-B/B' proteins were not detectably cleaved during anti-Fas induced apoptosis (Figure 2C).

The U1 snRNA molecule is cleaved during anti-Fas induced apoptosis

The results presented above strongly suggest that the Fas-mediated apoptotic cleavage of the U1-70K protein takes

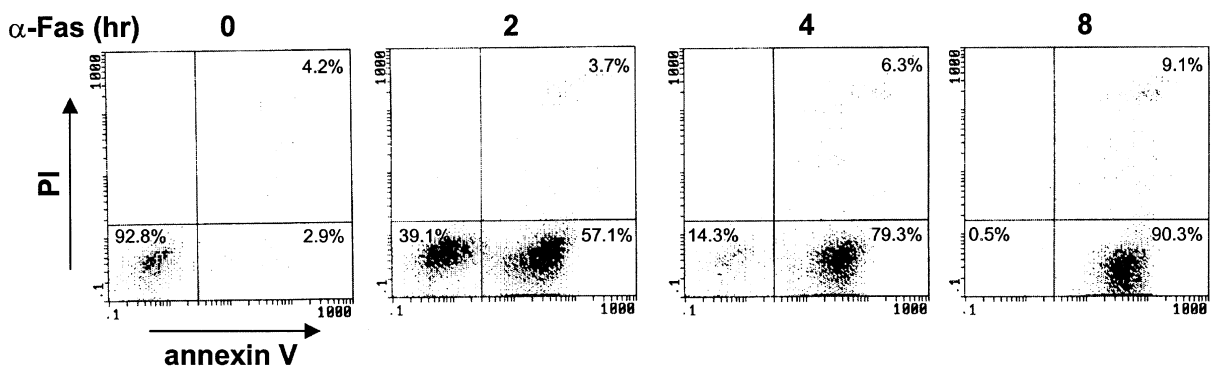


Figure 1 Induction of Fas mediated apoptosis in Jurkat/control cells by treatment with the monoclonal anti-Fas antibody 7C11 monitored by flow cytometry. PI, propidium iodide

place within the intact U1 snRNP complex. Since it was unclear how the U1 snRNA itself behaved during apoptosis, we performed additional experiments aimed at elucidating this issue. The behavior of the U1 snRNA molecule during anti-Fas induced apoptosis was examined by Northern blotting. Total RNA of Jurkat cells (Jurkat/control, Jurkat/Bcl-2) was isolated at various time points after the addition of anti-Fas antibody and subsequently analyzed by Northern blotting using an U1 snRNA specific probe. The results demonstrated the appearance of a somewhat faster migrating, truncated RNA species during apoptosis, which efficiently hybridized with the U1 snRNA specific probe, concomitant with a slight

decrease in the full-length U1 snRNA signal (Figure 3A, left panel). The analysis of this material on higher resolution gels (see also below) revealed that the truncated U1 snRNA species migrated as a doublet suggesting that the U1 snRNA is cleaved at two closely spaced sites. The first appearance of both shortened U1 snRNA products coincided with the first appearance of the 40-kDa cleavage product of the U1-70K protein, approximately between 2 and 4 h after the addition of anti-Fas antibodies (compare the left panels of Figure 2A, Figure 3A and Figure 6A). Quantitative measurements indicated that approximately 20–25% of the U1 snRNA molecules was cleaved 8 h after the induction of apoptosis in Jurkat/control cells. To confirm that cleavage of the U1 snRNA molecule in Jurkat cells incubated with the anti-Fas antibody was Fas receptor mediated, we also treated Jurkat/control cells with murine Fas-Ligand (FasL). As shown before with the anti-Fas antibody, the U1 snRNA molecule and the U1-70K protein were cleaved when FasL containing supernatant was used to induce apoptosis (Figure 3B). The cleavage products generated in both apoptotic scenarios appeared to be identical. Consistent with the retarded induction of apoptosis in the Jurkat/Bcl-2 cell line, the faster migrating U1 snRNA molecules appeared later and in smaller amounts (Figure 3A, right panel). The specificity of apoptotic U1 snRNA cleavage was substantiated by the lack of detectable changes in electrophoretic mobilities and quantities of U2, U3, U4, U5, and U6 snRNAs during the time period of apoptosis studied (Figure 3C).

Antibodies directed to several other snRNP components, such as U1A (mAb 9A9), U1-70K (mAb 2.73) and Sm-B/B', Sm-D1 and Sm-D3 (mAb Y12), immunoprecipitated both human U1 snRNA and its two cleavage products, indicating that these U1 snRNP-associated proteins remain associated with intact and cleaved U1 snRNA (results not shown).

Apoptotic cleavage of U1 snRNA is sensitive to caspase inhibition

It has been reported that in normal lymphocytes Bcl-2 does not inhibit Fas induced apoptosis²⁶ and that Bcl-2 can have different effects on cell death in the same cell depending on the apoptotic stimulus.²⁷ In the Bcl-2 overexpressing Jurkat transfectant tested in our experiments apoptosis was markedly inhibited (see Figure 2A and Figure 3A). To exclude an apoptosis inhibiting behavior specific for this particular transfectant, and to determine whether caspase activation is required to induce apoptotic cleavage of U1 snRNA (see Figure 3A), Jurkat/control cells were cultured in the presence of four cell permeable tetrapeptide caspase inhibitors, Ac-YVAD-CMK, Z-DEVD-FMK, Z-IETD-FMK, or Z-LEHD-FMK (irreversible inhibitors of caspases 1, 3, 8 and 9, respectively), for 1 h at 37°C prior to incubation with anti-Fas antibodies for an additional 8 h. The cleavage of U1 snRNA was markedly inhibited with the caspase-1, -3, and -8 inhibitors at 20 μM (Figure 4, lanes 3–8, lower panel) as compared to the control (lane 2), whereas hardly any inhibition of U1 snRNA cleavage was observed in the presence of the caspase-9 inhibitor (Figure 4, lanes 9 and 10, lower panel). All caspase inhibitors tested inhibited the U1-70K cleavage (Figure 4, lanes 3–10,

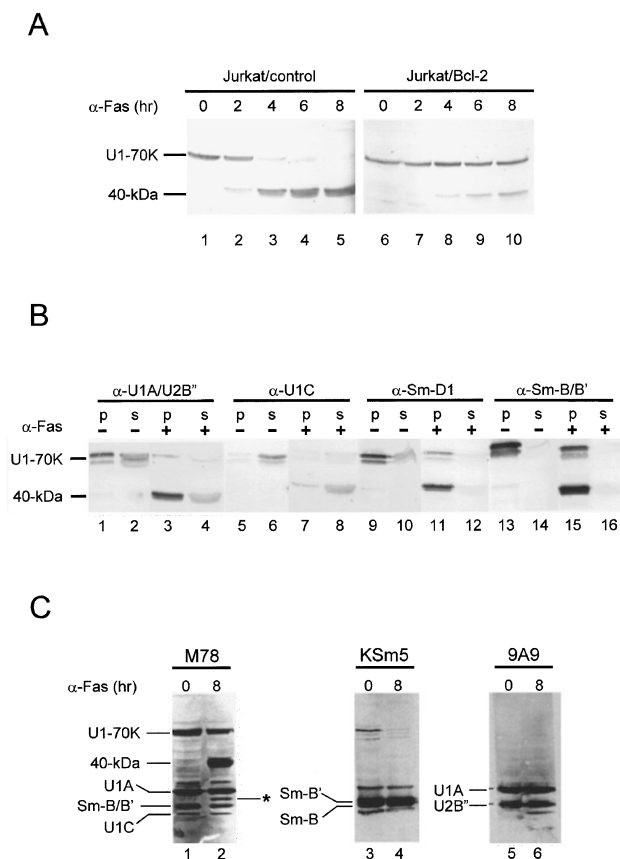


Figure 2 Cleavage of the U1-70K protein during anti-Fas induced apoptosis. (A) Jurkat/control and Jurkat/Bcl-2 cells were treated with the anti-Fas mAb 7C11 for the indicated time periods. Protein extracts were analyzed by 10% SDS-PAGE and Western blotting using an anti-U1-70K patient serum (H42). (B) The 40-kDa protein fragment of U1-70K remains associated with the U1 snRNP complex. U1 snRNP complexes were immunoprecipitated from non-apoptotic (–, 0 h, anti-Fas mAb) and apoptotic (+, 8 h, anti-Fas mAb) Jurkat/control cell extracts using anti-U1A/U2B' (α -U1A/U2B', mAb 9A9), anti-U1C (α -U1C, scFv K36), anti-Sm-D1 (α -Sm-D1, mAb KSm2) and anti-Sm-B/B' (α -Sm-B/B', mAb KSm5) monoclonal antibodies. Immunoprecipitates (p) and supernatants (s) were analyzed by 10% SDS-PAGE and Western blotting using an anti-U1-70K patient serum (H42). The positions of U1-70K and its 40-kDa cleavage product are indicated on the left. (C) U1 snRNP specific proteins are not detectably modified by cleavage. Jurkat/control cells were treated with anti-Fas mAb for the indicated time periods. Protein extracts were analyzed by 10% SDS-PAGE and Western blotting using anti-U1-70K/anti-U1A/anti-U1C/anti-Sm-B/B' patient serum (M78), and the anti-Sm-B/B' (mAb KSm5) and anti-U1A/U2B' (mAb 9A9) monoclonal antibodies. *C-terminal fragment of U1-70K after apoptotic cleavage

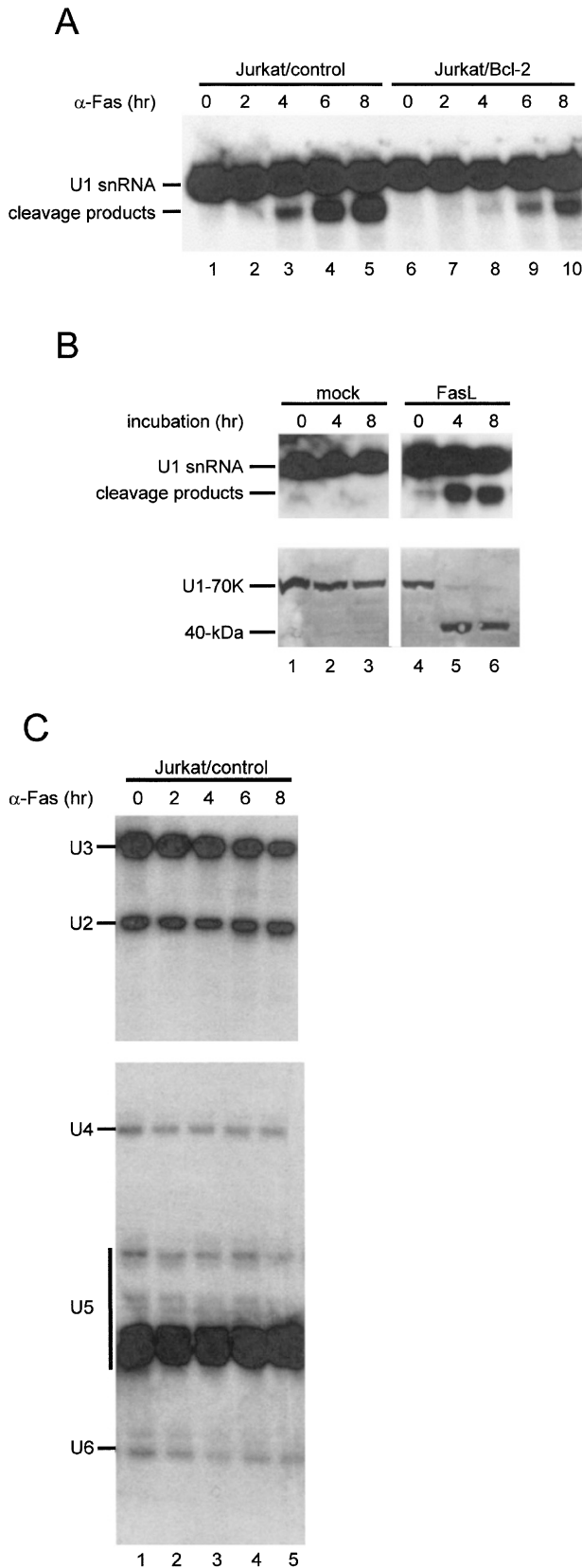


Figure 3 Cleavage of U1 snRNA during anti-Fas induced apoptosis. **(A)** The U1 snRNA molecule is cleaved during anti-Fas induced apoptosis. Jurkat cells were treated with the anti-Fas mAb 7C11 for the indicated time periods. Total

upper panel), although the inhibitory activity of the caspase-9 inhibitor was not as strong as that of the other inhibitors.

The U1 snRNA molecule is cleaved in other apoptotic cells

To investigate whether U1 snRNA cleavage in apoptotic cells is a universal phenomenon, we analyzed U1 snRNA by Northern blot hybridization in various other apoptotic cells and after the induction of apoptosis with different stimuli. The results show that the U1 snRNA molecule is cleaved in human HL-60 (promyelocytic leukemia) and in mouse 4B1 cells (WR19L cells overexpressing the human Fas-receptor)²⁸ treated with the anti-Fas antibody 7C11 (Figure 5, lanes 1–5), in Jurkat/control cells treated with staurosporine, cycloheximide or anisomycin (Figure 5, lanes 6–9), and in human HeLa (cervix carcinoma) and HL-60 cells treated with anisomycin (Figure 5, lanes 10–13). From these experiments it can be concluded that the cleavage of the U1 snRNA molecule is a general feature of the degeneration process in the apoptotic cell.

Apoptotically cleaved U1 snRNA lacks a 2,2,7-trimethylguanosine (TMG) cap

To analyze which part of the U1 snRNA molecule is removed during anti-Fas induced apoptosis, immunoprecipitations were carried out using monoclonal antibodies directed to the 2,2,7-trimethylguanosine (TMG) cap (H20) and to U1-stemloop II (Z5scFv3).²⁹ The anti-U1-stemloop II scFv antibody immunoprecipitated the U1 snRNA as well as its cleavage products (Figure 6A, upper panel). In contrast, the anti-TMG cap antibody precipitated full-length U1 snRNA but not its cleavage products, indicating that the TMG cap was absent from the latter (Figure 6A, lower panel). Although no electrophoretic mobility changes were detected in the U2–U6 snRNAs by Northern blot hybridization (Figure 3C), it could not be excluded that the TMG cap of these RNAs (except for U6 snRNA, which is not TMG capped) was also lost or changed during apoptosis. Therefore, RNAs immunoprecipitated with anti-TMG cap antibodies were analyzed by 3'-end labeling and denaturing gel electrophoresis. Like the full-length U1 snRNA, the U2, U3, U4 and U5 snRNAs could be precipitated with the anti-TMG cap antibody from apoptotic cell extracts, indicating that these RNAs were not de-capped (Figure 6B).

RNA was size fractionated on a 6% polyacrylamide/8M urea sequencing gel and analyzed by Northern blot hybridization using a ³²P-labeled antisense U1 RNA probe. The positions of full-length U1 snRNA and its cleavage products are indicated on the left. **(B)** The U1 snRNA molecule is cleaved during FasL induced apoptosis. Jurkat/control cells were treated with murine FasL for the indicated time periods and total RNA was analyzed by Northern blot hybridization as described in **A**. Total protein extracts were analyzed by 10% SDS-PAGE and Western blotting using an anti-U1-70K patient serum (lower panel). The positions of U1 snRNA and its cleavage products as well as U1-70K and its 40-kDa cleavage product are indicated on the left. **(C)** The U2, U3, U4, U5 and U6 snRNAs are not cleaved during anti-Fas induced apoptosis. Total RNA from Jurkat/control cells treated with the anti-Fas mAb 7C11 for the indicated time periods was analyzed by Northern blot hybridization as described in **A** using ³²P-labeled antisense U RNA probes as indicated on the left

Mapping of the apoptotic U1 snRNA cleavage site

Based on the finding that the cleaved U1 snRNA could not be precipitated via anti-TMG cap antibodies, S1 nuclease mapping was used to determine the identity of the 5'-end(s) of the cleaved U1 snRNA. A 49 nucleotides long 5'-end ³²P-labeled oligonucleotide complementary to the 5'-end of the wild type (native) U1 snRNA was incubated overnight with deproteinized total RNA from Jurkat/control and apoptotic cells, followed by digestion with S1 nuclease. The first (5') 39 nucleotides of this oligonucleotide are complementary to the 5'-end of the wild type U1 snRNA molecule, whereas a stretch of ten A-residues completed the oligonucleotide at the 3'-end (Figure 7A). Post-transcriptional modifications in the most 5'-end of the native U1 snRNA molecule (i.e. a 2,2,7-trimethylguanosine (TMG) cap, a 2'-O-methylated adenosine and uridine, and two pseudouridines (Ψ), appeared to destabilize the DNA-RNA hybrid. As a consequence, the region of the probe corresponding to the terminal region of native U1 snRNA was more sensitive to S1 nuclease digestion resulting in several (minor) nuclease protection products in addition to

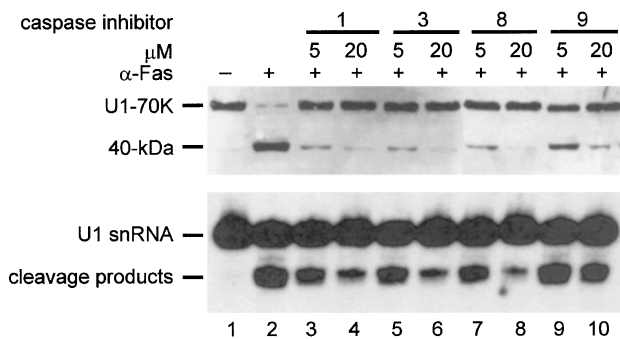


Figure 4 Apoptotic cleavage of U1 snRNA is sensitive to caspase inhibition. Jurkat/control cells were incubated with four cell permeable tetrapeptide caspase inhibitors, 5 μM (lanes 3, 5, 7, 9) or 20 μM (lanes 4, 6, 8, 10) of caspase-1, -3, -8 and -9 inhibitors, for 1 h at 37°C prior to incubation with anti-Fas antibodies for an additional 8 h (+). Total protein extracts were analyzed by 10% SDS-PAGE and Western blotting using an anti-U1-70K patient serum (upper panel). Total RNA was analyzed by Northern blot hybridization using a ³²P-labeled antisense U1 RNA probe (lower panel). In lanes 1 and 2 material from non-apoptotic (-, 0 h, anti-Fas mAb) and apoptotic (+, 8 h, anti-Fas mAb) cells, respectively, was analyzed. The positions of U1-70K and its 40-kDa cleavage product as well as U1 snRNA and its cleavage products are indicated on the left

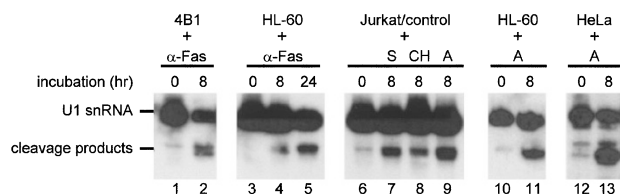


Figure 5 The U1 snRNA molecule is cleaved in various apoptotic cells treated with different apoptotic stimuli. Cells were treated with anti-Fas mAb 7C11, staurosporine (S), cycloheximide (C) or anisomycin (A) for the indicated time periods. Total RNA was isolated and analyzed by Northern blot hybridization using a ³²P-labeled antisense U1 RNA probe. The positions of full-length U1 snRNA and its cleavage products are indicated on the left

products corresponding to the 5'-ends of intact (39 nt) and cleaved U1 snRNA (<39 nt) (Figure 7B, lanes 1 and 2). The precise lengths of the various S1 protected oligonucleotide fragments were determined using 5'-end ³²P-labeled oligonucleotides as marker. The longest S1 protected oligonucleotide contains 40 nucleotides (Figure 7B, lanes 1 and 2) and corresponds to the S1 protected 5'-end of the complete U1 snRNA (39 nt) plus the first flanking A-residue (A₄₀). The inability of S1 nuclease to remove A₄₀ efficiently is probably the result of steric effects by and possibly unusual base-pairing with the 2,2,7-trimethylguanosine cap of intact U1 snRNA. The S1 protected oligonucleotide fragments of 33 and 34 nucleotides resulting from U1 snRNA immunoprecipitated from apoptotic cell extracts (Figure 7B, lane 2) were much more intense than the corresponding fragments resulting from non-apoptotic RNA (Figure 7B, lane 1). This strongly suggested that these protected oligonucleotide fragments corresponded to the 5'-ends of the apoptotically cleaved U1 snRNA molecules. This was confirmed in an experiment in which full-length and cleaved U1 snRNA were gel purified (6% polyacrylamide/8 M urea) and independently analyzed by nuclease protection. The 33 and 34 nucleotides long S1 protected oligonucleotide fragments indeed correspond to the 5'-ends of the cleaved U1 snRNA molecules (Figure 7B, lanes 3 and 4). Thus the results of the S1 mapping experiment indicate that the U1 snRNA molecule is cleaved either between nucleotides C5 and Ψ6, or between nucleotides Ψ6 and Ψ7.

Discussion

During the last 5 years there has been a growing interest in finding possible connections between apoptosis and the induction of autoimmunity. At present, many autoantigens have been found that are modified during apoptosis,¹³ and it has been hypothesized that the recognition, uptake, processing, or presentation of these modified self-antigens may promote autoantibody production.

One of these autoantigens is the U1-70K protein, a component of the U1 small nuclear ribonucleoprotein (U1 snRNP) complex. This protein (437 aa)³⁰ has an important function in the pre-mRNA splicing reaction as it enhances the interaction of the U1 snRNP complex with the 5' splice site. In 1994, Casciola-Rosen and collaborators¹⁶ reported that the U1-70K protein was specifically cleaved during apoptosis, and they subsequently demonstrated that the U1-70K protein was cleaved at position ³³⁸DGPD³⁴¹ by caspase-3 (CPP32, Apopain, Yama), resulting in an N-terminal 40-kDa (aa 1-341) and a C-terminal 22-kDa (aa 342-437) protein fragment.¹⁷ By analyzing the U1 snRNP complex during anti-Fas induced apoptosis, we demonstrated that it is likely that the U1-70K protein is cleaved while it is associated with the intact U1 snRNP complex in apoptotic cells, and that the N-terminal 40-kDa fragment remains associated with the U1 snRNP complex. The latter finding is consistent with the location of the RNA-binding domain (RBD, aa region 104-184) in the N-terminal half of the molecule, which has been shown previously to be sufficient for U1 snRNP association.³¹ No obvious apoptotic

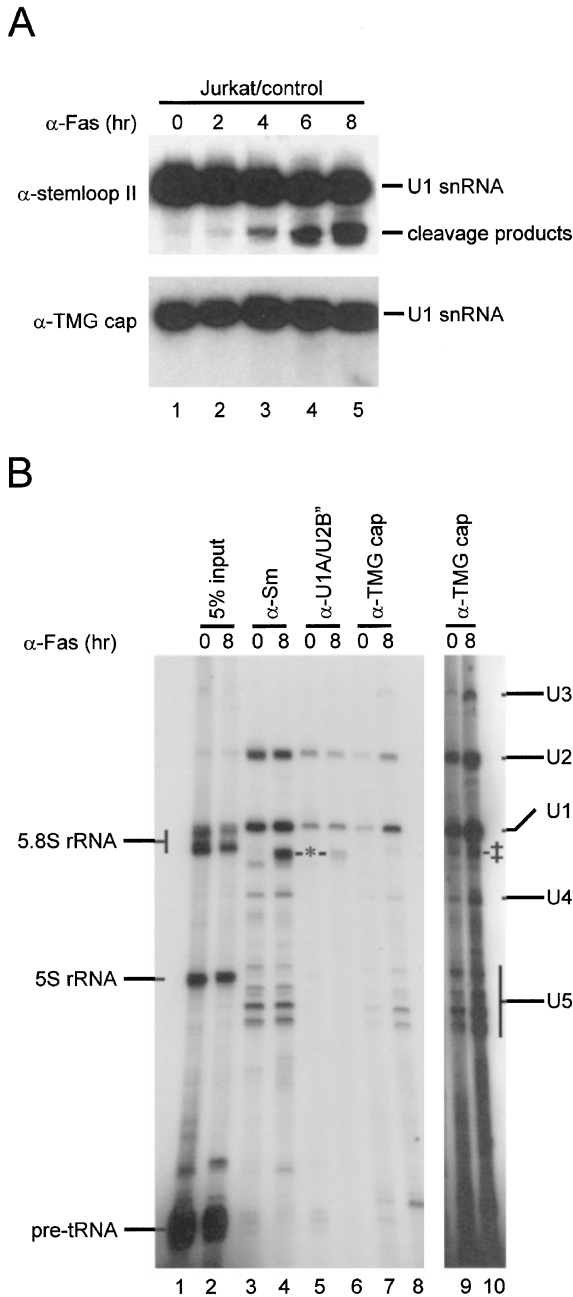


Figure 6 The U1 snRNA molecule loses its TMG cap structure during anti-Fas induced apoptosis. **(A)** Apoptotically cleaved U1 snRNA can not be precipitated with anti-TMG cap antibodies. Jurkat cells were treated with the anti-Fas mAb 7C11 for the indicated time periods. U1 snRNP complexes were immunoprecipitated from cell extracts using anti-stemloop II (scFv Z5scFv3, upper panel) and anti-TMG cap (mAb H20, lower panel) antibodies. Immunoprecipitated RNA was size fractionated on a 6% polyacrylamide/8M urea sequencing gel and analyzed by Northern blot hybridization using a ³²P-labeled antisense U1 RNA probe. The positions of full-length U1 snRNA and its cleavage products are indicated on the right. **(B)** The U2, U3, U4 and U5 snRNAs remain TMG capped during apoptosis. U snRNP complexes were immunoprecipitated from non-apoptotic (0 h. anti-Fas mAb) and apoptotic (8 h. anti-Fas mAb) Jurkat/control cell extracts using anti-Sm (*x*-Sm, mAb Y12), anti-U1A/U2B' (*x*-U1A/U2B', mAb 9A9), and anti-TMG cap (*x*-TMG cap, mAb H20) antibodies. Immunoprecipitated RNA was ³²P-pCp labeled, size fractionated on a 10% polyacrylamide/8M urea sequencing gel and visualized by autoradiography. Lanes 9 and 10 show an overexposure of lanes 7 and 8. The positions of the most abundant RNAs in the cell extracts (input, lanes 1

changes were detected in the U1 snRNP associated proteins U1A, U1C, and the Sm-proteins B/B' (²⁵ and Figure 2C).

The major finding of the present study is that the U1 snRNA molecule is specifically cleaved in apoptotic mammalian and mouse cells, either between nucleotides C5 and Ψ6 or between nucleotides Ψ6 and Ψ7, resulting in

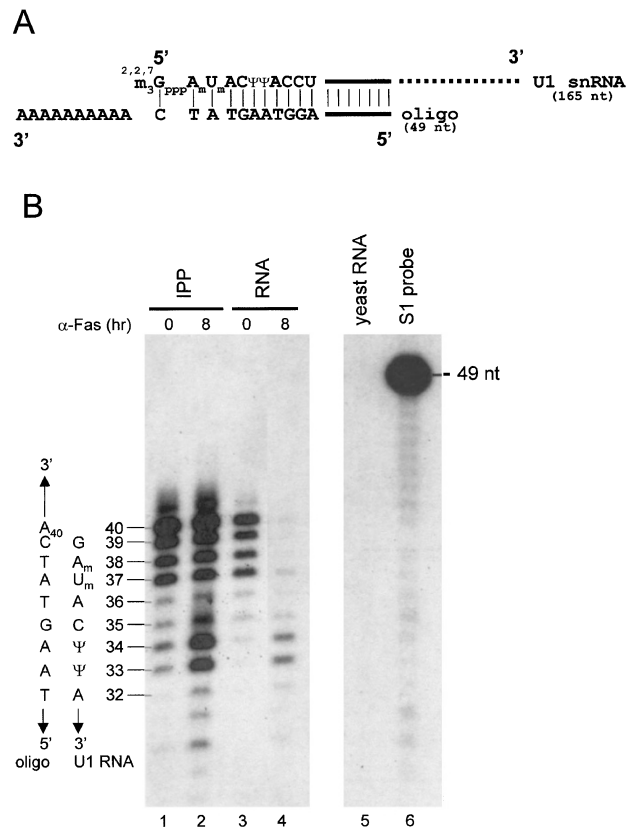


Figure 7 S1 nuclease mapping of the apoptotic U1 snRNA cleavage site(s). **(A)** Schematic structure of the 5'-end of U1 snRNA hybridizing with the 49 nucleotides long oligonucleotide that was used as a probe for nuclease protection. Note the presence of the TMG cap, methylated riboses at positions 2 and 3 and pseudouridines (Ψ) at positions 6 and 7 in the 5'-terminal region of native U1 snRNA. **(B)** Nuclease S1 protection data for apoptotic U1 snRNA. The 49 nucleotides long ³²P-labeled oligonucleotide complementary to the 5'-end of the U1 snRNA molecule was hybridized with RNA from non-apoptotic (0 h. anti-Fas mAb) or apoptotic (8 h. anti-Fas mAb) Jurkat/control cells (equivalent of 5 × 10⁶ cells), or with yeast RNA (as a control). Upon treatment with nuclease S1, the reaction products were analyzed by denaturing gel electrophoresis. Lane 1, immunoprecipitated non-apoptotic U1 snRNA; lane 2, immunoprecipitated apoptotic U1 snRNA; lane 3, gel purified native U1 snRNA; lane 4, gel purified apoptotically cleaved U1 snRNA; lane 5, yeast RNA at 37°C (as a control); lane 6, the 49 nucleotides long undigested ³²P-labeled oligonucleotide. The immunoprecipitations were performed with the anti-U1A/U2B' monoclonal antibody 9A9. On the left the size of the protected fragments is indicated, as well as the corresponding nucleotides of both the oligonucleotide probe and U1 snRNA

and 2) and of the U snRNAs are indicated on the left and on the right, respectively. *Apoptotic cleavage products of U1 snRNA. ‡Degradation products of U1 snRNA only seen after overexposure

two truncated apoptotic RNA molecules each starting with a pseudouridine (Ψ). These results might suggest that pseudouridines are involved in the selection of cleavage sites for the apoptotically activated nuclease responsible for U1 snRNA cleavage. However, the mere presence of Ψ -residues in single-stranded regions of RNAs does not seem to be sufficient for cleavage, since the U2, U3, U4, U5, and U6 snRNAs are not cleaved despite the presence of numerous Ψ -residues.^{32,33} Also the presence of two consecutive Ψ -residues, which occur at the cleavage sites of U1 snRNA, does not seem to constitute the cleavage determinant, because such a Ψ -doublet also occurs in a single-stranded region near the 5'-end (nucleotides 7 and 8) of the U2 snRNA molecule, which apparently is not cleaved at this position. Taken together, while at least one pseudouridine (in a single-stranded region) might be required for recognition by the putative apoptotically activated RNase, other structural elements seem to be necessary for cleavage to occur. Identification and characterization of the involved ribonuclease will be required to learn more about the substrate specificity of this enzyme. The U1 snRNA molecule is the first small RNA with a known cellular function reported to be cleaved during apoptosis. So far, only the cleavage of two ribosomal RNAs, i.e. the 16S mitochondrial rRNA and the 28S rRNA, have been reported.^{34,35} Mapping of the apoptotic 16S mitochondrial rRNA cleavage sites has not been performed, but in view of our data it would be interesting to investigate whether in these cases Ψ -residues are also involved. For the 28S rRNA, fine mapping has been performed, but Ψ -residues seemed not to be involved.³⁶ Furthermore, we recently observed that the Ro RNP associated Y RNAs, the biological function of which is unknown, are rapidly degraded in apoptotic cells.³⁷

The post-transcriptional modifications of U into Ψ observed at highly conserved positions in snRNAs and also other RNAs, like tRNAs and rRNAs, are in the U snRNAs mainly found in single-stranded segments involved in RNA-RNA interactions.^{38–40} Although the function of Ψ -residues in U snRNAs remains unknown, in U1, U2, and U5 snRNA, these Ψ s are present in regions known to be critically important for the function of these snRNPs in pre-mRNA splicing (for a review see³³). Removal of the first 5–6 nucleotides including the TMG cap of the U1 snRNA molecule will certainly thwart pre-mRNA splicing, although data to support this are not (yet) available. Interestingly, it has been shown that oligonucleotides complementary to the first 14 nucleotides of the U1 snRNA molecule can block pre-spliceosome and spliceosome assembly *in vitro*.⁴¹ We showed that the complete cellular pool of U1-70K molecules is cleaved into the 40-kDa fragments, whereas only a subfraction (20–25%) of the U1 snRNA molecules is cleaved 8 h after Fas-induced apoptosis. Due to the instability and morphological changes of apoptotic cells, it is very difficult to establish which part, cytoplasmic or nuclear, of the U1 snRNA population is cleaved during apoptosis. A subset of U1 snRNPs might be protected from nuclease cleavage, either by association with other proteins (e.g. SR proteins or other spliceosomal proteins), or by sequestration in a separate cellular compartment, that is

inaccessible for the nuclease. Most probably, only the U1 snRNA of non-spliceosomal free U1 snRNP is cleaved.

One of the most important biochemical events after the induction of apoptosis is the activation of the caspase cascade. Although the exact order of caspase activation is not known yet, it has been established that activation of caspase-3 can be induced via the activation of several other caspases, including caspase-1, -8, and 9. Inhibition of Fas-induced apoptosis with caspase-1, -3, -8, or 9 inhibitors, showed that the U1 snRNA cleavage was markedly inhibited in the presence of the caspase-1, -3 and -8 inhibitors. It should be stressed that although these tetrapeptide inhibitors display a (strong) preference for a particular caspase, their specificity is not absolute and at relatively high concentrations other caspases will also be inhibited. Therefore, the present results do not allow conclusions on the caspase that is responsible for activation of the nucleolytic activity that cleaves U1 snRNA. On the one hand the caspase inhibition data would be consistent with caspase-3 being involved in activation of this nuclease, but on the other hand the incomplete inhibition of U1 snRNA cleavage under conditions where cleavage of a model substrate for caspase-3 (U1-70K) is virtually completely inhibited argues against this possibility. These results thus seem to indicate that the U1 snRNA molecule is cleaved by an apoptosis specific ribonuclease that is not (directly) activated by caspase-1, -3, -8 or 9. The fact that this ribonuclease seems to affect only the U1 snRNA molecule and not the U2, U3, U4, U5 or U6 snRNAs is intriguing.

Autoantibodies reactive with components of the U1 snRNP complex are specifically found in the sera of patients with SLE. The translocation of the U1 snRNP complex from its normal subcellular localization into apoptotic bodies near the surface of cells undergoing apoptosis, might facilitate exposure of the immune system to such antigens when clearance of apoptotic cell remnants is somehow disturbed. In combination with the apoptotic modifications of U1 snRNP components, i.e. cleavage of the U1-70K protein, association of hyperphosphorylated SR proteins, and cleavage of the U1 snRNA molecule itself, this might bypass immunological tolerance. All these structural alterations and modifications might be important for the production of autoantibodies reactive with the U1 snRNP complex, and support the idea of a connection between apoptosis and the emergence of autoimmunity.

Materials and Methods

Cell lines

Jurkat (human T cell leukemia) suspension cells were grown in RPMI-1640 medium (Gibco-BRL) supplemented with 10% heat inactivated fetal calf serum (FCS) (Gibco-BRL), sodium-pyruvate (1 mM), penicillin (1 mM) and streptomycin (1 mM), and were maintained at a concentration of 1×10^6 cells/ml. Jurkat cells transfected with Bcl-2 (Jurkat/Bcl-2) (a gift of John Reed, La Jolla, CA, USA) or with the empty transfection vector (Jurkat/control) and 4B1 cells (mouse WR19L cells overexpressing the human Fas-receptor)²⁸ were cultured

in the same medium with the addition of 200 $\mu\text{g/ml}$ G418 (Gibco-BRL). HeLa (human cervix carcinoma) cells and HL-60 (human promyelocytic leukemia) cells were grown in DMEM (Gibco-BRL) supplemented with 10% FCS, penicillin and streptomycin. Murine Neuro2A cells transfected with murine Fas-Ligand (FasL) (kindly provided by Dr. A Fontana, Zürich, Switzerland) or with the empty transfection vector (mock) were cultured in DMEM supplemented with 5% FCS, penicillin, streptomycin and 200 $\mu\text{g/ml}$ G418.⁴² Cell lines were grown in a humidified 37°C incubator containing 5% CO₂.

Induction of cell death

To induce apoptosis, Jurkat suspension cells were seeded at a concentration of $1 \times 10^6/\text{ml}$ and incubated either with: the monoclonal anti-Fas antibody 7C11 (a gift of Michael Robertson, Indiana University, Bloomington, IN, USA) at a final dilution of 1:500 as described,⁴³ with murine FasL or mock supernatant (see below), with 10 $\mu\text{g/ml}$ anisomycin, with 0.5 μM staurosporine or with 100 $\mu\text{g/ml}$ cycloheximide. HL-60 and 4B1 cells were treated with the anti-Fas antibody 7C11, HeLa cells and HL-60 cells were treated with 10 $\mu\text{g/ml}$ anisomycin. Cells were incubated at 37°C for the indicated time periods before harvesting. After induction of cell death, cells were washed three times with PBS and used immediately or stored at -70°C.

For experiments utilizing the cell permeable tetrapeptide caspase inhibitors (Calbiochem), Jurkat cells (1.5×10^6 cells/ml) were cultured for 1 h at 37°C in the presence of 5 and 20 μM Ac-YVAD-CMK, Z-DEVD-FMK, Z-IETD-FMK, or Z-LEHD-FMK (irreversible inhibitors of caspases 1, 3, 8 and 9, respectively) in DMSO, prior to the induction of apoptosis with the monoclonal anti-Fas antibody 7C11. To minimize spontaneous cell death, cells were maintained at a concentration of 1×10^6 cells/ml by diluting every 2–3 days in fresh medium to maintain exponential cell growth.

Production of murine Fas-Ligand (FasL)

Murine FasL was obtained from murine FasL-transfected Neuro2A cells after 2 days of culture in DMEM supplemented with 5% FCS without G418. Supernatant was collected and cleared from cells and debris by centrifugation and used to induce apoptosis in Jurkat/control cells as described above. Supernatant from mock-transfected Neuro2A cells was used as control. Cells were incubated at 37°C for the indicated time periods before harvesting. After induction of cell death, cells were washed three times with PBS and used immediately or stored at -70°C.

Flow cytometry

Induction of apoptosis with the monoclonal anti-Fas antibody 7C11 in Jurkat/control cells, was monitored by staining the cells with annexin V-FITC (1 $\mu\text{g/ml}$) in RPMI, 10% FCS, 2.5 mM CaCl₂ for 2 min at room temperature, followed by propidium iodide (5 $\mu\text{g/ml}$) for 10 min at 4°C. Staining was determined using a Coulter Epics Elite flow cytometer.

Preparation of cell extracts and Western blot analysis

Cells were lysed on ice for 30 min in NP-40 lysis buffer (25 mM Tris-HCl, pH 7.5, 1% NP-40, 100 mM KCl, 10 mM MgCl₂, 0.25 mM DTE, containing the Complete protease inhibitor cocktail (Boehringer Mannheim)). Cell lysates were centrifuged for 15 min at 4°C (13000 r.p.m.) and the supernatants were used immediately or

stored at -70°C. Protein extracts of 1×10^6 cells were size-fractionated by 10% SDS-PAGE and blotted onto nitrocellulose filters (Schleicher & Schuell). Western blots were blocked in washing buffer (5% skimmed milk, 50 mM Tris, 150 mM NaCl, 0.5% Tween-20) and subsequently incubated either with: SLE patient serum H42 (anti-U1-70K) or M78 (anti-U1-70K, anti-U1A, anti-U1C, anti-Sm-B/B') diluted 1:5000 in washing buffer, or with culture supernatant (diluted 1:10 in washing buffer) of one of the following mouse monoclonal antibodies: 9A9 (anti-U1A/U2B'),²² or KSm5 (anti-Sm-B/B').²⁴ Blocking and antibody incubation were each performed for 1 h at room temperature. After extensive washing (4 times 10 min), blots were incubated with peroxidase-conjugated rabbit anti-human Ig antibodies (Dako) or with peroxidase-conjugated rabbit anti-mouse Ig antibodies for 1 h at room temperature. After washing (4 times 10 min), bound secondary antibodies were visualized via chemoluminescence.

Patient serum samples

The serum samples H42 and M78 were provided by patients seen at the Department of Rheumatology and Internal Medicine of the Academic Hospital Nijmegen, The Netherlands. Diagnoses were reached as described by de Rooij et al.⁴⁴

Immunoprecipitation

Protein A-agarose beads (20 μl of 50% slurry, Kem-En-Tec) were coated with 200 μl culture supernatant of one of the following mouse monoclonal antibodies: 9A9 (anti-U1A/U2B'),²² KSm2 (anti-Sm-D1),²⁴ KSm5 (anti-Sm-B/B'),²⁴ Y12 (anti-Sm-B/B'/D1/D3),⁴⁵ 2.73 (anti-U1-70K)⁴⁶ or with 1 ml culture supernatant of mouse monoclonal anti-VSV-G-tag antibody, which was used to immobilize VSV-G-tagged human scFv antibody fragments. Since the anti-VSV-G-tag mAb was of the IgG1 isotype, the beads were precoated with rabbit anti-mouse IgG (Dako) for 2 h at 4°C. The periplasmic fractions of tagged scFv anti-U1C (K36)²³ and scFv anti-U1-stemloop II (Z5scFv3)²⁹ were coupled to protein A-agarose via the anti-VSV-G-tag antibodies for 2 h at 4°C. Anti-2,2,7-trimethylguanosine (TMG) cap antibodies (20 μg of H20, Euro-Diagnostica BV) were coupled to 20 μl protein A-agarose. Unless stated otherwise, all incubations were performed overnight at 4°C in IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% NP-40) by end-over-end rotation. Between incubations, beads were washed three times with IPP150. Immunoprecipitations were carried out with 20 μl of a 1×10^8 cell equivalents/ml Jurkat extract in IPP150 by end-over-end rotation for 2 h at 4°C and subsequently washed three times with IPP150. The beads were either resuspended in 100 μl IPP150/0.5% SDS to isolate co-immunoprecipitated RNA via TRIzol (see below), or in 10 μl SDS sample buffer to analyze protein complexes via SDS-PAGE and Western blotting. To analyze non-immunoprecipitated proteins after immunoprecipitation, the supernatant was precipitated with 5 volumes of acetone for 1 h at -70°C. After spinning for 15 min (13000 r.p.m.) at 4°C, the samples were dried, dissolved in SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

RNA isolation and Northern blot analysis

RNA was isolated by direct addition of TRIzol reagent (Gibco-BRL) to cell lysates (see above) or immunoprecipitated U1 snRNP complexes, according to the manufacturer's specifications. RNA was size fractionated on 6% polyacrylamide/8 M urea gels, blotted onto Hybond N⁺ filters (Amersham) and hybridized as described previously.⁴⁷ Northern blots were washed with $2 \times \text{SSC}/1\%$ SDS

(three times 20 min at 65°C) and analyzed by autoradiography. In case of cell lysates, the total RNA of 0.5×10^6 cells was size fractionated.

Probes

To obtain antisense ^{32}P -labeled human (h) U1, hU3, hU4 and hU6 RNA probes, the corresponding DNA constructs were linearized and 1 μg was transcribed with either SP6 or T7 RNA polymerase in the presence of ^{32}P -UTP essentially as described before.⁴⁸ *Xenopus laevis* (x) U2 and hU5 DNA probes for hU2 snRNA and hU5 snRNA detection, were obtained after ^{32}P -dCTP labeling of inserts isolated from the corresponding DNA constructs, using a random primed DNA labeling kit according to the manufacturer's instructions (Boehringer Mannheim).

pCp labeling

Immunoprecipitated RNA was 3'-end labeled using ^{32}P -pCp and T4 RNA ligase as described before.⁴⁹

Determination of the U1 snRNA cleavage site via nuclease protection

Nuclease S1 mapping was performed as described before⁵⁰ with some minor modifications. U1 snRNA (supplemented with 100 μg yeast RNA) and yeast RNA (as control) were annealed with 2.5×10^4 c.p.m. of the 5'-end ^{32}P -labeled antisense oligonucleotide 5'-CCTTCGTGATCATGGTATCTCCCCTGCCAGTAAGTATC(A)₁₀-3' at 40°C overnight. The first 39 nucleotides of this oligonucleotide are complementary to the 5'-end of the human U1 snRNA sequence. The ten A-residues at the 3'-end of the oligonucleotide were added to generate a protruding 3'-end in the DNA-RNA hybrid. After overnight annealing, the non-hybridized regions of the oligonucleotide were digested by incubation with 1000 units/ml nuclease S1 (Boehringer Mannheim) for 1 h at 37°C. The remaining products were electrophoresed in a 10% polyacrylamide/8 M urea gel, which was subsequently exposed for autoradiography. As additional size markers, several 5'-end ^{32}P -labeled oligonucleotides were used. Non-apoptotic and apoptotic Jurkat cell extracts or total RNA were used to isolate U1 snRNA either via immunoprecipitations with the monoclonal antibody 9A9 (anti-U1A/U2B^{''}),²² or via 6% polyacrylamide/8 M urea gel electrophoresis followed by RNA elution. All 5'-end ^{32}P -labeled oligonucleotides were isolated by PAGE-purification. RNA and oligonucleotides were eluted from the gels by adding 200 μl elution buffer (500 mM NH₄Ac, pH 6.5, 10 mM MgCl₂, 0.1% SDS, 0.5 $\mu\text{g}/\mu\text{l}$ yeast RNA) to the gel slices and incubating overnight at 4°C.

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