Volume 16 Number 23 1988 Nucleic Acids Research

Direct cross-linking of snRNP proteins F and 70K to snRNAs by ultra-violet radiation *in situ*

Andreas Woppmann, Jutta Rinke and Reinhard Lührmann*

Max-Planck-Institut für Molekulare Genetik, Otto Warburg Laboratories, D-1000 Berlin 33, FRG

Received September 27, 1988; Revised and Accepted November 8, 1988

ABSTRACT

Protein-RNA interactions in small nuclear ribonucleoproteins (UsnRNPs) from HeLa cells were investigated by irradiation of purified nucleoplasmic snRNPs U1 to U6 with UV light at 254 nm. The cross-linked proteins were analyzed on one- and two-dimensional gel electrophoresis systems, and the existence of a stable cross-linkage was demonstrated by isolating protein-oligonucleotide complexes from snRNPs containing ^{32}P-labelled snRNAs after exhaustive digestion with a mixture of RNases of different specificities. The primary target of the UV-light induced cross-linking reaction between protein and RNA was protein F. It was also found to be cross-linked to U1 snRNA in purified U1 snRNPs. Protein F is known to be one of the common snRNP proteins, which together with D, E and G protect a 15-25 nucleotide long stretch of snRNAs U1, U2, U4 and U5, the so-called domain A or Sm binding site against nuclease digestion (Liautard et al., 1982). It is therefore likely that the core-protein may bind directly and specifically to the common snRNA domain A, or else to a sub-region of this. The second protein which was demonstrated to be cross-linked to snRNA was the U1 specific protein 70K. Since it has been shown that binding of protein 70K to U1 RNP requires the presence of the 5' stem and loop of U1 RNA (Hamm et al., 1987) it is likely that the 70K protein directly interacts with a sub-region of the first stem loop structure.

INTRODUCTION

Eucaryotic cells contain a group of small nuclear RNAs, of which the principal members are the snRNAs U1, U2, U4, U5 and U6 (reviewed in [1-3]). These are found in the nucleoplasm in the four ribonucleoprotein particles (snRNPs) U1, U2, U5 and U4/U6 [4,5]; they have sedimentation coefficients from 9S to 12S [6-8] and a density of 1.43 g/cm^3 in CsCl gradients [9]. Protein analysis of the snRNPs (reviewed in [10]) led to the identification, besides the seven proteins B', B, D, D', E, F and G common to these particles [11-16], of several particle-specific polypeptides. Thus, U1 RNP possesses in addition the proteins 70K, A and C; U2 contains the proteins A' and B", and U5 may contain a 25K protein [11-18]. Proteins specific to the U4/U6 RNP have not yet been identified.

The snRNPs appear to play an essential role in the processing of nuclear pre-mRNA (reviewed in [19-22]). There is experimental evidence for the participation of all the major snRNPs U1-U6 in the splicing of pre-mRNAs (e.g., [23-33]). Suggestively, the snRNP proteins are needed for the particles to carry out their task of splicing [34-36]. However, the

exact functions of the various snRNP particles in the individual steps of the splicing process, which takes place in the so-called 50S-60S "spliceosome" [37-39], have yet to be elucidated.

In recent years, many experimental approaches have yielded information about the principles of snRNP structure (reviewed in [40]). Thus, the snRNAs U1, U2, U4 and U5 possess a common structural motif, the so-called A domain [41]. This consists of the short single-stranded consensus sequence $PuA(U)_nGPu$, where n>3, termed "region *i*", and two flanking hairpin loops with stems that vary in length among the snRNAs. In addition, the 3'-localised hairpin loop contains the less strongly conserved sequence PyNPyG [42]. In the remaining, nuclease-resistant RNP structures of the various snRNP particles, a 15-25-nucleotide-long stretch, containing region *i*, has been identified by nuclease protection experiments. This stretch is complexed with the common proteins D, E, F and G [43]. In the course of snRNP assembly [40], these four proteins appear already to form an RNA-independent 6S protein hetero-oligomer in the cytoplasm [44].

Most of the UsnRNP proteins are - to differing extents - antigens for the so-called anti-RNP and anti-Sm autoantibodies, that are frequently produced by patients with rheumatoid connective tissue diseases [7,45]. The corresponding sera can therefore be used *inter alia* as tools for the structural investigation of the snRNPs. While anti-Sm sera contain antibodies against the common snRNP proteins, and therefore immunoprecipitate all the snRNPs U1-U6, anti-RNP sera contain only antibodies against U1 snRNP-specific proteins. Immunoprecipitation tests combined with nuclease-protection, sequence-deletion and sequence-substitution experiments reveal the association of the common snRNP proteins with region *i* (e.g. [43,46-48]). However, it has not yet been possible to show which of the proteins of the D, E, F, G "core" interact(s) directly with this region, so that the $PuA(U)_nGPu$ region is often termed the Sm binding site. It is assumed that the common proteins B and B' bind to the snRNA protein "core" complex [48,49]. Analogous experiments have shown that the two 3'-localised hairpin loops of the U2 snRNA are necessary for the binding of the U2 RNP-specific polypeptides A' and B" [48]. Furthermore, it has recently been shown that the 5' "stem-loop" structure of U1 snRNA is needed for the association of this particle with the U1-specific polypeptides 70K and A [49]. However, for the particle-specific proteins as well as for the common ones, it is still unclear which proteins interact directly with the snRNA and which are associated with it by way of protein-protein interaction.

A possible means of identifying direct protein-RNA binding is the covalent stabilisation of this interaction by cross-linking, followed by analysis of the cross-linked products. The use of short-wavelength ultra-violet light (λ = 254 nm) has been found to be particularly effective for the stabilisation of direct RNA-protein interactions, since the induction of a covalent binding requires that the groups to be cross-linked are not further apart than one covalent bond length (reviewed in [59]). Such studies have included the cross-linking of DNA and DNA polymerase [50], *E. coli* tRNA and aminoacyl-tRNA synthetase [51], *E. coli* rRNA

and ribosomal proteins (e.g. [52], reviewed in [53]), hnRNA and hnRNP proteins [54-56] and mRNA and polysomal proteins [57,58]. In addition, UV absorption by proteins has a minimum at λ = 254 nm, while RNA has a 50-fold stronger maximum at 260 nm, so that irradiation doses at 254 nm weak enough to leave the RNA undamaged will neither degrade the proteins nor efficiently cross-link them with one another. (This may be contrasted, for example, with irradiation at 280 nm, at which wavelength protein-protein cross-links dominate [60].) In this work, we have demonstrated by the use of this "zero length cross-linker" that there is a direct interaction between snRNA and the snRNP "core" protein F, and between snRNA and the U1-RNP-specific polypeptide 70K.

MATERIALS AND METHODS

Preparation of snRNPs

The cultivation of HeLa S3 cells in suspension culture, their labelling with [^{32}P]-orthophosphate and the preparation of nucleoplasmic extracts were carried out as described by Bringmann *et al.* [61]. snRNPs U1-U6 were isolated from nuclear extracts by immunoaffinity chromatography with the monoclonal anti-m_3G antibody H20 [62] by a method based on that of Bringmann *et al.* [63]. Pure U1 RNPs were obtained from this snRNP mixture by chromatography on DEAE-Sepharose columns [16]. The snRNPs were dialysed against a buffer containing 20 mM Tris.HCl pH 7.0, 2 mM $MgCl_2$, 50 mM NH_4Cl (TMN) before use in all experiments.

UV irradiation

In order to avoid absorption of the snRNPs on the walls of the irradiation cell in the TMN buffer, 0.05% (v/v) NP40 was added. 200-μl samples (10 - 100 μg/ml) were irradiated at 4°C in plastic dishes of diameter 15.5 mm (area 189 mm^2) and a "solution depth" of 1.0 mm. The irradiation was at wavelength 254 nm with a Sylvania G8T5 germicidal lamp (USA) and with the doses stated; these were calibrated with a UV-dosimeter (80X OPTOMETER^{TM}, Unit. Det. T., Inc., USA).

Determination of RNA-protein cross-linking

The 3' terminus of the RNA in U1 snRNPs dialysed against TMN was labelled radioactively by overnight incubation at 4 °C with cytidine-3',5'-[5'-^{32}P]-biphosphate ([^{32}P]-pCp, 3 μCi/pmol) at a molar ratio of pCp:RNA of 2:1 (24 μCi [^{32}P]-pCp/μg U1 RNP) in the presence of 3.3 mM DTE, 40 μM ATP and 20 - 50 units of T4 RNA ligase (PL Biochemicals, USA). Per dose, 2 μg [^{32}P]-pCp-U1 RNP was irradiated; the RNP was then precipitated by the addition of 0.1 volume 4 M sodium acetate (pH 5.6) and 5 volumes 95% ethanol at -20 °C, and washed once with 80% ethanol at -20 °C. Before precipitation, control samples were withdrawn and incubated with 0.5% SDS (w/v) and 10 μg proteinase K (Merck, FRG) for 30 min at 37 °C.

The ethanol-precipitated samples were dried *in vacuo* and dissolved in 7 μl sample buffer

(100 mM Tris borate, pH 8.3, 8 M urea, 1% w/v SDS, 0.1% w/v bromphenol blue, 0.1% w/v xylene cyanol). After denaturation at 65 °C for 15 min, they were analysed by polyacrylamide gel electrophoresis in the presence of 7 M urea and 0.1% (w/v) SDS (gel size 400 x 210 x 0.5 mm). Electrophoresis at 4 °C with 800 V applied to the gel was continued until the xylene cyanol tracking dye had migrated to the bottom of the gel. HaeIII-restricted pBR322 DNA, whose 5' termini had been labelled with $\gamma[^{32}P]$-ATP and T4 polynucleotide kinase (Calbiochem, USA), was used as a length standard for autoradiography. An autoradiography film was exposed to the undried gel; the bands detected were excised from the gel and counted in a scintillation counter. Where stated, they were then eluted in a buffer containing 10 mM Tris.HCl (pH 7.0), 0.05% w/v SDS, 6 mM 2-mercaptoethanol and 1 mM EDTA.

Isolation of snRNP proteins by treatment with nuclease

In order to guarantee complete hydrolysis of RNA down to a fragment size not larger than 3 nucleotides, up to 20 µg of snRNPs U1 - U6 in ca. 200 µl TMN buffer were treated with nuclease in several digestion steps along the lines described previously by Stiege et al. [64]. 1µg RNase A (Boehringer, FRG), 2,5 µg RNase T1 (PL Biochemicals, USA) and 5 µg nuclease S7 (micrococcal nuclease; Boehringer) were added to the samples. The first incubation was carried out at 37 °C for 45 min in the presence of 10 mM $CaCl_2$. After this, either 0.5% w/v SDS was added and the incubation was continued for a further 45 min, or urea was added in two steps to give concentrations of 4 M and 7 M urea, respectively, followed by incubation at 37 °C for 20 min after each addition. The temperature was then raised gradually over a further 45-min period from 37 °C to 60 °C. After cooling to 37 °C, the pH value of the sample was shifted from 7.0 to 4.5 (the optimum for RNase T2) by the addition of 0.2 M sodium acetate. Residual oligonucleotides were hydrolysed with 5 µg RNase T2 (Calbiochem) for 45 min at 37 °C and then for a further 45 min during which the temperature was raised gradually from 37 °C to 60 °C. The proteins were precipitated by the addition of 9 volumes of acetone in cases where SDS was present, or of 5 volumes of 95% v/v ethanol in cases where urea was present, at -20 °C. Subsequently they were washed with 80% v/v ethanol at -20 °C and analysed after drying.

Identification of snRNP phosphoproteins

The proteins of snRNPs U1 to U6 labelled *in vivo* with [^{32}P]-orthophosphate were isolated either by the addition of 0.5% w/v SDS, extraction with 1 volume PCA (phenol/chloroform/isoamyl alcohol 50:49:1) and precipitation with a 5-fold excess of acetone, or else by nuclease treatment and acetone precipitation (see above) followed by washing in 80% ethanol and analysis in a 15 % discontinuous SDS-polyacrylamide gel (Laemmli, [65]). After staining with Coomassie R250, the gel was dried and autoradiographed.

In certain cases, phosphoryl serine moieties (as described by Cooper et al. [66]) were hydrolysed. For this purpose, the dried and autoradiographed gel was swollen in fresh

1 M potassium hydroxide solution and then, after removal of the Whatman 3MM paper, heated for 2 h at 55 °C with gentle shaking. The alkaline solution was then replaced by a solution of acetic acid (10%) and methanol (40%), and the gel was shaken gently until, after several changes of the acid solution, it had shrunk back to its original size. It was again stained with Coomassie blue, dried and autoradiographed.

Analysis of proteins cross-linked to RNA

2 x 10^5 or 6 x 10^5 cpm of snRNPs U1 to U6 were irradiated at 254 nm with a dose of 18 mJ/mm^2, either alone or after the addition of 20 µg of unlabelled U1 - U6 snRNPs. The snRNP proteins were isolated by nuclease treatment and analysed either on discontinuous SDS polyacrylamide gels (see above), or else by two-dimensional gel-electrophoresis, using *non-equilibrium-pH-gradient-electrophoresis* (NEPHGE) within a pH 3-10 gradient in gel rods as the first dimension, and SDS gel electrophoresis as the second [10; A.W., P. Bringmann, F. Godt and R.L., manuscript in preparation].

Where stated, the autoradiographically detected protein bands were excised from the gel and the gel pieces were soaked for 2 h in a solution containing 2% w/v SDS and 400 mM NH_4HCO_3 (pH 7.9); after dilution with water to give 0.4% SDS and 80 mM NH_4HCO_3, the proteins were eluted by shaking overnight. In the case of the related controls the eluted proteins were digested for 1 h at 37 °C with 5 µg proteinase K in the presence of 0.5% w/v SDS.

The analysis of isolated U1 RNPs (UV-irradiated or non-irradiated) was carried out by immunoprecipitating these from 1 x 10^6 cpm [^{32}P]-snRNPs U1 - U6 plus 20 µg unlabelled U1 - U6 snRNPs by the use of human anti-(U1)RNP serum P.83, the antibodies of which had been coupled covalently with dimethyl suberimidate to protein A Sepharose [67]. The precipitate was treated with nucleases (see above) and the proteins were dissolved in denaturing elution buffer (100 mM Tris.HCl at pH 6.8, 250 mM sucrose, 10 mM EDTA, 100 mM DTE, 5% w/v 2-mercaptoethanol, 2.5% w/v SDS and 0.01% w/v bromphenol blue) at 100 °C for 5 min. The eluate was loaded directly onto discontinuous SDS gels (see above) and electrophoresed.

Limited proteolysis of the gel-eluted [^{32}P]-phospho-70K protein plus 4 µg of electroeluted, non-labelled 70K was carried out by first removing the SDS contained in the lyophilised eluate by a single washing with 100 ml 90% (v/v) acetone, 5% acetic acid and 5% triethanolamine followed by two washings with 95% ethanol chilled to -20 °C, and then by treatment with chymotrypsin A4 ($m_{(70k)}$:$m_{(chym.)}$ = 15:1; Boehringer, FRG) for 30 min at 37 °C [68].

Demonstration of the cross-linked 70K-U1 RNA complex

6-µg samples of the U1 snRNPs were incubated in ca. 60 µl TMN buffer containing 1 mM DTE, 0.5 mM PMSF and 9 µCi γ[^{32}P]-ATP (0.5 µCi/pmol) for 1 h at 37 °C, under which conditions the kinase activity that co-chromatographs with the snRNPs specifically

phosphorylates protein 70K (see Results and Discussion). The sample volumes were made up to 200 µl with TMN buffer and then, after addition of 0.05% v/v NP40, either irradiated with a 14-mJ/mm^2 dose of 254-nm UV light or kept on ice without irradiation. An irradiated control sample was incubated with 10 µg RNase T1 for 15 min at 37 °C.

After acetone precipitation and washing with 80% ethanol, the dried samples were dissolved in denaturing sample buffer (50 mM Tris.HCl, pH 6.8, 5% w/v 2-mercaptoethanol, 2% w/v SDS, 10% glycerol, 0.01% w/v BPB) by warming for 10 min to 90 °C. They were then analysed on an 8-15% SDS-polyacrylamide gradient gel (stacking gel: 5% polyacrylamide).

RESULTS AND DISCUSSION

I) Determination of the extent of RNA-protein cross-linking

For the induction of covalent cross-links between amino acids and nucleic acids, the radiation used in previous studies [reviewed in 53,59] has usually been at a wavelength of 254 nm and a dose of 10-40 mJ/mm^2. We therefore began by determining the optimal dose at λ = 254 nm, this being defined by the best obtainable compromise between a high yield of covalent RNA-protein cross-links and avoidance of degradation of the RNA. Our standard method of analysis was to label the 3' ends of the RNA of isolated U1 RNPs from HeLa cells, then to irradiate them, and finally to analyse them by electrophoresis on a 10% polyacrylamide gel containing 0.1% sodium dodecylsulphate and 7 M urea (see Materials and Methods).

Two examples of such dose dependence studies are shown in Figure 1. As the carrier of the radioactive label is the U1 RNA, RNA-protein complexes can be identified as such by their sensitivity to proteinase K. Two RNA-protein complexes termed K I and K II are detected in minor amounts without UV treatment, and irradiation increases the quantities observed up to tenfold. The increased yield after irradiation is a consequence of the stabilisation by UV-induced covalent attachment of U1 RNA to proteins in direct contact with it. However, it appears that the non-covalent complexes are very stable; otherwise they would not withstand the denaturing conditions used (15 min at 65 °C in sample buffer containing 7.2 M urea and 1% SDS).

In addition, a small amount of a UV-induced U1 RNA internal cross-link, identified by its resistence to proteolysis, is seen (Figure 1B), but this does not interfere with the following identification of proteins cross-linked to snRNA.

The autoradiograms in Figure 1 show substantial degradation of RNA at doses exceeding 22,5 mJ/mm^2. We therefore chose doses from 9 to 18 mJ/mm^2 to induce covalent bonds between snRNAs and the snRNP proteins that bind them.

It was necessary to confirm that the complexes K I and K II are products of a specific RNA-protein cross-linking reaction. The integrity of the RNA component of the complexes

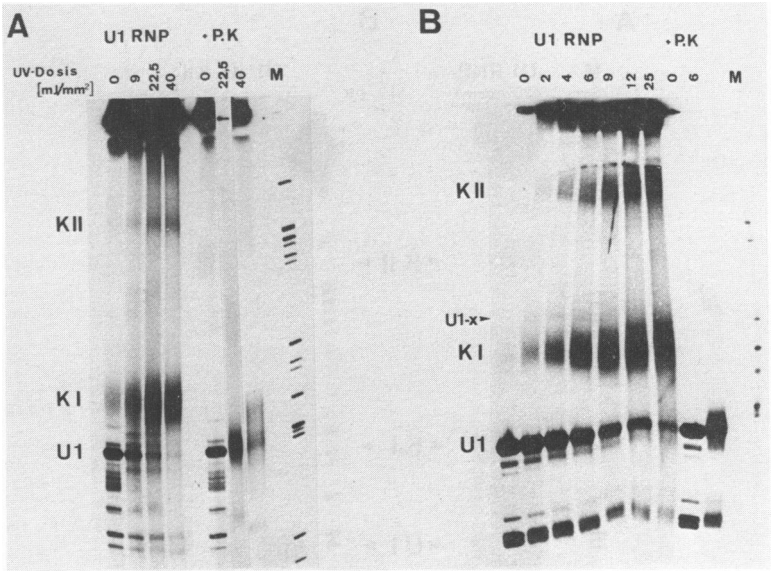

Fig. 1. *SDS/urea gel electrophoresis of UV-irradiated U1 snRNPs.* Equal amounts of [^{32}P]-pCp-labelled U1 RNPs were irradiated at λ = 254 nm with the doses indicated and then applied to the 10% gel as described in Materials and Methods. In the case of the +P.K controls, the samples were digested with proteinase K after irradiation and before analysis on the gel; this allowed identification of the RNA-protein complexes by inspection of their labelled RNA component. The positions of the free U1 RNA and of the detected RNA-protein complexes K I and K II are indicated. Radioactive pBR322 DNA digested with HaeIII was used as a length standard (M). In (B), a part of the dose range shown in (A) was tested in small steps. U1-x is internally cross-linked U1 RNA (see Results).

was confirmed by extracting the two complexes (as prepared by irradiation at 9 mJ/mm^2) from the polyacrylamide gel and subjecting them to repeated electrophoresis both with and without digestion by proteinase K. The autoradiograms of the primary, preparative gel and the second, analytical gel are shown in Figure 2. Apart from some aggregation, the undigested complexes migrate to the same position on the second gel as on the first. Digestion with proteinase K releases the RNA, which then migrates to the same position as does the RNA from non-irradiated U1 snRNP. All the evidence thus points strongly to the formation of specific, UV-inducible covalent bonds between snRNA and snRNP proteins in direct contact.

II) Identification of the proteins cross-linked to snRNA

The technique of choice for the identification of the snRNP proteins cross-linked to the snRNA is termed "nucleotide transfer". In this method, the RNA is [^{32}P]-labelled *in vivo* along its entire length and, after complex formation by irradiation, the snRNPs are digested with a mixture of RNases of different specificities (for details see Materials and Methods). This leaves the proteins intact with a small oligonucleotide (e.g., dinucleotide) attached. The

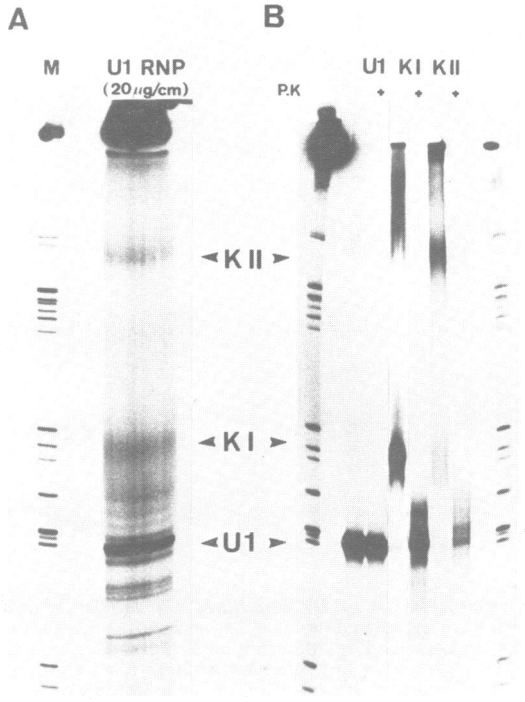

Fig. 2. *Electrophoretic analysis of the U1 RNP internal complexes K I and K II.* (A) 40 µg [^{32}P]-pCp-labelled snRNPs were irradiated at 254 nm with a dose of 9 mJ/mm^2 and then subjected to preparative SDS/urea gel electrophoresis. The complexes K I and K II and the non-cross-linked U1 RNA were eluted from the gel as described in Materials and Methods. M = marker as in Figure 1. (B) The eluates were divided into two portions. In each case one portion was treated with proteinase K, and the samples were applied to the SDS/urea gel. Left-hand lane in each case, undigested; right-hand lane (+), digested.

proteins are then analysed by discontinuous SDS-polyacrylamide gel electrophoresis or by two-dimensional gel electrophoresis, in which the cross-linked proteins are detectable as radioactive bands or spots, respectively.

In the application of this technique to snRNPs, the first step was to check whether any of the snRNP proteins are phosphorylated *in vivo* before cross-linking.

a) Investigation of snRNP proteins for phosphorylation

snRNPs were labelled *in vivo* with [^{32}P]-orthophosphate. 2 x 10^5 cpm or 6 x 10^5 cpm of snRNPs U1-U6 were added to unlabelled snRNPs U1-U6, to give a total protein mass in each case of 20 µg. The proteins were isolated either by extraction with PCA or by digestion with the nucleases A, T1, S7 and T2, and afterwards the proteins were analysed on a discontinuous SDS gel (see Materials and Methods).

Figure 3 demonstrates that the only strongly phosphorylated snRNP protein is the protein 70K. In addition to this electrophoretic evidence, we have confirmed that the carrier of the

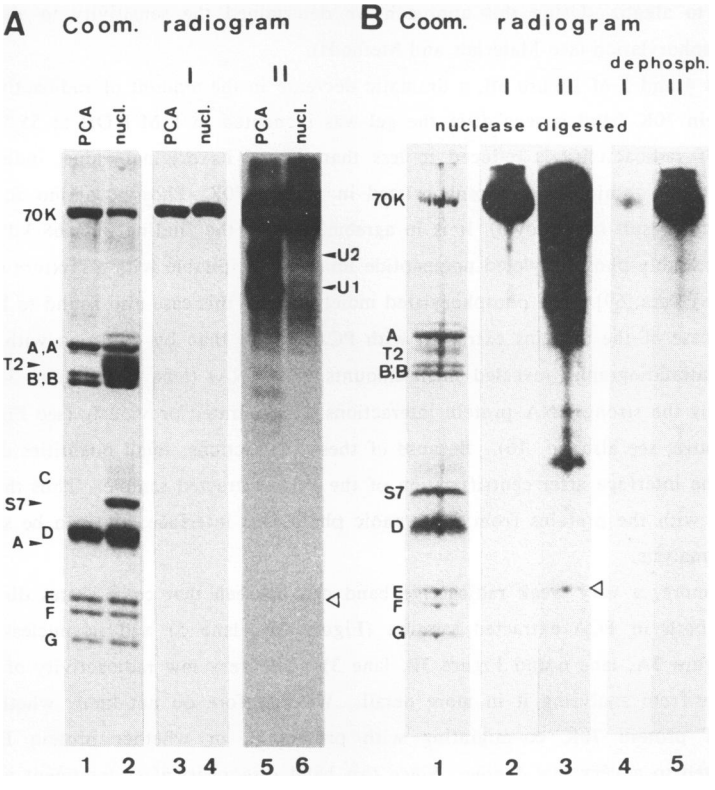

Fig. 3. *snRNP phosphoproteins*. The proteins of snRNPs U1-U6 labelled in vivo, mixed with unlabelled snRNPs to give 20 μg total protein, were isolated either by PCA extraction or by intense nuclease digestion and analysed on a discontinuous SDS gel as described in Materials and Methods. I and II indicate respectively short and long exposures of the film.
A: starting activity of sample 2 x 10^5 cpm. Lanes 1, 3, 5, PCA extraction; lanes 2, 4, 6, nuclease digestion. Lanes 1 and 2 show the result of Coomassie blue staining (the positions of migration of snRNP proteins and enzymes are indicated on the left) and the lanes 3-6 the result of autoradiography. U1, U2, positions of co-precipitated snRNA; arrowheads, phosphoprotein co-migrating with protein E (see text).
B: starting activity of sample 6 x 10^5 cpm, RNA removed by nuclease digestion. Lane 1, Coomassie blue staining; lanes 2, 3, autoradiography; lanes 4, 5, the same lane of gel as in 1-3 but after dephosphorylation of serine with alkali (see Materials and Methods).

radioactive label is protein 70K by various experiments, e.g. by a radioimmune precipitation assay with a 70K-specific monoclonal antibody (not shown). Measurement of the amount of radioactivity on the 70K band showed that 1% of the total activity of the sample was associated with this protein, indicating that 70K is a heavily phosphorylated protein.

The three phosphoamino acids phosphoserine, phosphothreonine and phosphotyrosine have different sensitivities to alkali, which provides a simple method if distinguishing between them [66]. While the phosphoester bond in phosphoserine is hydrolysed by more than 75% under the conditions stated, phosphotyrosine is hydrolysed by less than 1%. Phosphothreonine

is resistant to alkali. Using this approach we determined the sensitivity to alkali of the protein phosphorylation (see Materials and Methods).

In lanes 4 and 5 of Figure 3B, a dramatic decrease in the amount of radioactive material in the protein 70K band is seen after the gel was incubated in 1 M KOH at 55 °C for 2 h. The level of radioactivity is reduced to less than 1% of its original value, indicating that serine is the only amino acid phosphorylated in protein 70K. Phospho amino acid analysis confirmed this result (not shown). It is in agreement with the finding of a 68 kd protein as the only detectably phosphorylated polypeptide immunoprecipitable with a mixture of anti-Sm and anti-RNP sera [69]. The phosphorylated moiety was in this case also found to be serine.

In the case of the proteins extracted with PCA (rather than by digestion with nuclease), prolonged autoradiography revealed small amounts of snRNAs (lane 5 in Figure 3A). These are caused by the strong RNA-protein interactions demonstrated previously (see Figure 1 and Section I above; see also ref. 16). Because of these interactions, small quantities of snRNAs remain in the interface after centrifugation of the PCA-extracted sample. Thus they are co-precipitated with the proteins from the organic phase plus interface, and can be seen in the subsequent analysis.

Furthermore, a very weak radioactive band can be seen that co-migrates directly with protein E, both in PCA-extracted samples (Figure 3A, lane 5) and in nuclease-digested samples (Figure 3A, lane 6 and Figure 3B, lane 3). The very low radioactivity of this band prevented us from analysing it in more detail. We therefore do not know whether it is a fragment of protein 70K co-migrating with protein E, or whether protein E itself is phosphorylated to a very low degree. Since this band disappears after treatment with alkali, the phosphorylated residue is again serine.

None of the other radioactive bands visible after prolonged exposure migrates at the same position as any other snRNP protein. As protein 70K is known to be relatively quickly degraded, these bands are probably fragments of protein 70K.

b) Nucleotide transfer analysis of non-phosphorylated proteins

With protein 70K and possibly protein E identified as the only phosphorylated snRNP proteins, the next step was to try to detect proteins in direct contact with their associated RNA, inducing cross-links by UV irradiation and analysing the products by nuclease digestion and electrophoresis. If the digestion is carried out with a mixture of nucleases A, T1, S7 and T2, as described in Materials and Methods, the RNA component is degraded to fragments no larger than three nucleotides, although the snRNPs, irradiated or not, are more or less in their native conformations when the nucleases are added (see also ref. 64).

The suitability of the nuclease cocktail was investigated in a series of preliminary experiments. The efficiency of the digestion was confirmed by electrophoresis of the digestion products. It was shown that the nucleases are not contaminated by protease activity, and that there was no possibility that RNA fragments co-migrated with snRNP proteins in the

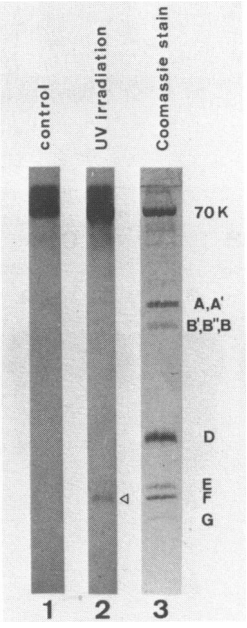

Fig. 4. *Cross-linking of protein F to snRNAs upon UV irradiation of intact snRNPs.* 2 x 10^5 cpm U1-U6 labelled *in vivo* with ^{32}P were irradiated at 254 nm with a dose of 18 mJ/mm^2 (lane 2); an identical control sample was stored on ice (lane 1). Thereafter, samples were digested with nucleases (see Materials and Methods) and electrophoresed on a discontinuous SDS-polyacrylamide gel alongside 15 μg of unlabelled snRNP proteins (lane 3). The autoradiogram of lanes 1 and 2 beside the Coomassie-stained lane 3 shows that nucleotide is transferred to protein F only (arrowhead).

discontinuous SDS gels (15% polyacrylamide in the separating gel), even in the size range of the small proteins E, F and G.

Figure 4 shows a typical analysis of the proteins of snRNPs U1-U6 after the snRNPs, ^{32}P-labelled *in vivo*, had been irradiated at 254 nm with a dose of 18 mJ/mm^2 and subsequently treated with the nuclease mixture. As expected from its normally phosphorylated state, protein 70K gives a strong band both in the non-irradiated control (lane 1) and after irradiation (lane 2).

The only radioactive band seen in the irradiated sample and not in the non-irradiated one migrates at a rate corresponding to protein F (compare the Coomassie-stained marker proteins in lane 3). This finding was substantiated in several ways in subsequent experiments, the results of which are shown in Figures 5 and 6. Here, the amount of radioactive snRNPs (made up to 20 μg with unlabelled snRNPs) was three times higher.

First of all, apart from the phosphorylation signals from protein 70K, the presumed 70K fragments and possibly protein E (cf. Figure 3) visible in the lane of the non-irradiated

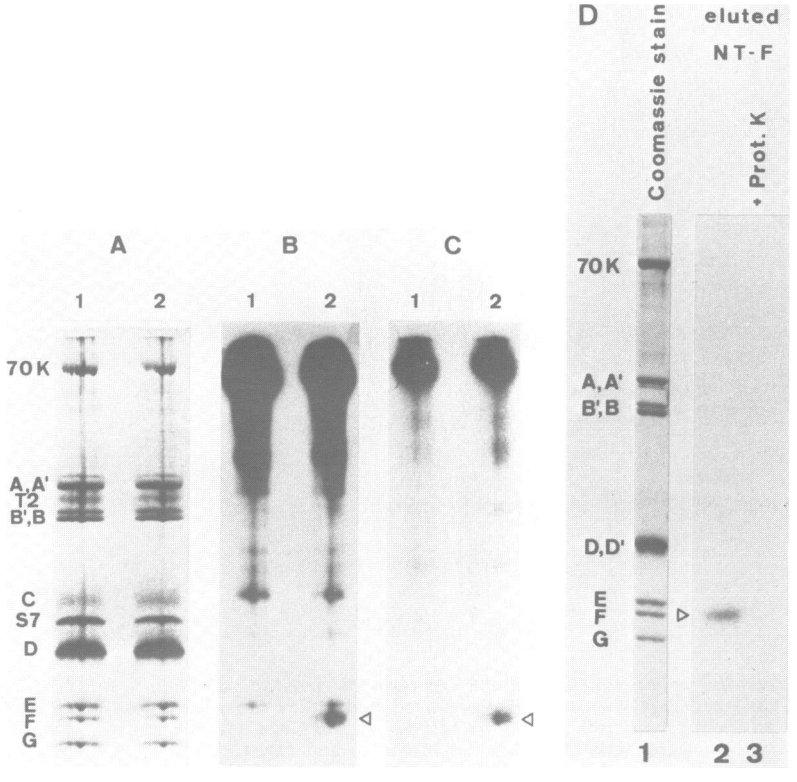

Fig. 5. *Effects of phosphopeptide dephosphorylation and of protease treatment on the cross-linked band associated with protein F.* 6 x 10^5 cpm snRNPs labelled with ^{32}P *in vivo* were mixed with 20 μg unlabelled snRNPs. They were then irradiated at 254 nm with a dose of 18 mJ/mm² (lane 2), while an identical control sample was stored on ice without irradiation (lane 1). Both samples were then digested with nucleases and analysed by discontinuous SDS-polyacrylamide gel electrophoresis (see Materials and Methods).
A: Coomassie-stained gel; positions of snRNP proteins and nucleases are shown.
B: An autoradiogram of the same gel.
C: An autoradiogram of the same gel after alkaline hydrolysis of phosphoserine residues (see text).
D: The radioactive protein F band (ca. 300 cpm) was eluted from an unfixed gel. The eluate was divided into two parts, of which one was treated with proteinase K, and both were electrophoresed on an SDS gel, in parallel with 20 μg snRNP proteins as length standards and with subsequent autoradiography. Arrowhead: the eluted protein F band (lane 2), which disappears after proteolysis (lane 3).

control (lane 1 in Figure 5A and B), the only detectable UV-induced radioactive band again corresponds directly to the position of protein F (lane 2 in Figure 5A, B and C).

Secondly, the UV-induced band is insensitive to treatment by alkali. This is seen by comparing Figures 5B (before) and 5C (after). The signals due to the phosphorylated proteins decrease considerably during alkali treatment, whereas that corresponding to protein F remains unaffected, which indicates that protein F is labelled in a fundamentally different

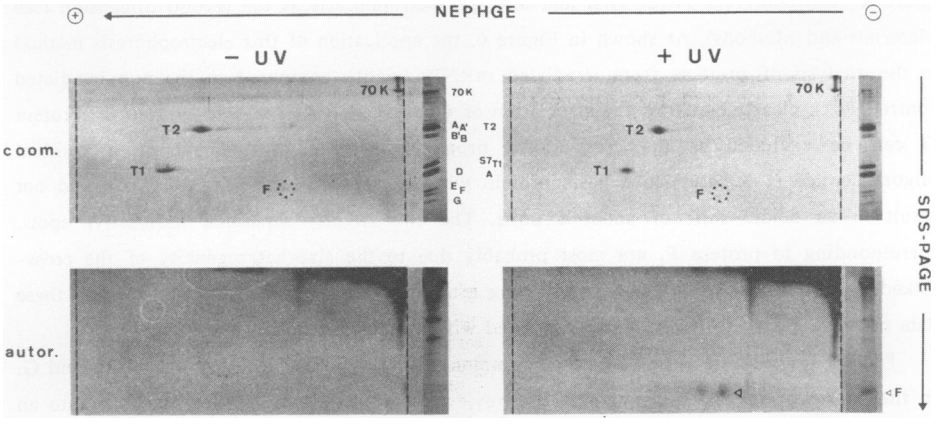

Fig. 6. *Two-dimensional separation of protein F cross-linked to snRNA.* 1 x 10^6 cpm [^{32}P]-snRNPs U1-U6, mixed with 20 μg unlabeled snRNPs were irradiated as in Fig. 5 and digested with nucleases in the presence of 7 M urea instead of SDS. The isofocussing gels contained 9 M urea and 1 % (v/v) carrier ampholytes pH 3-10 (Servalyte). Proteins were applied to the basic end of the focussing dimension (It should be noted that, owing to their basic pI values all other snRNP proteins, with the exception of proteins F and 70K, do not enter the gel when applied to the basic end). In the second dimension the proteins were fractionated in a 15 % SDS-polyacrylamide gel (see Materials and Methods). The upper panels display Coomassie Blue stained 2-D gels, the lower panels the corresponding autoradiograms.
On the right side of each panel total proteins from snRNPs U1 to U6 treated the same way as the sample used for two-dimensional separation were applied directly to the second dimension gel (15 % SDS gel). In the case of the irradiated samples (+UV) the nucleotide transfer signals corresponding to protein F can be seen in the autoradiogram.

way. In particular, the UV-generated band cannot be due to degradation or modification by irradiation of one of the existing phosphoproteins. It is in any case generally accepted that proteins are not affected by mild irradiation at 254 nm (see Introduction).

Thirdly, the radioactively labelled species continues to migrate with protein F after extraction from the first gel and application to a second (Figure 5D, lane 2), and it is destroyed by digestion with proteinase K (Figure 5D, lane 3). This confirms the proteinaceous nature of the substance that is radioactively labelled by UV irradiation.

Since protein F is not normally phosphorylated, and the UV-induced band is not sensitive to serine-specific dephosphorylation in alkali, the only possible explanation for the specific UV-induced radioactive labelling of this protein is that it is cross-linked to the snRNA.

The new radioactive band clearly co-migrates with protein F, but the possibility that it is in fact another protein must be considered. The only candidate for this is protein G, since the attached oligonucleotide would tend to retard the parent protein. This possibility could be discarded, however, by the following experiment.

Because of its extremely acidic isoelectric point of pI ≤ 4 [10], protein F can be separated from all other snRNP-proteins by two-dimensional electrophoresis, using *non-equilibrium-pH-*

gradient-electrophoresis as the first and SDS gel elctrophoresis as the second dimension (see Materials and Methods). As shown in Figure 6, the application of this electrophoresis method to the analysis of proteins from irradiated snRNPs (right), compared to the non-irradiated control (left), clearly confirms the association of the radioactive signal with protein F. Protein G can be excluded as the cross-linked protein, since an attachement of a residual oligonucleotide (1-3 bases) to a basic protein with an apparente pI-value of 9.6 would not result in an acidic shift of about 6 units. The two slightly separated radioactive spots, corresponding to protein F, are most probably due to the size-heterogeneity of the cross-linked oligonucleotides (1-3 bases) remaining after nuclease digestion. Taken together, these data prove that protein F is directly associated with the snRNA in intact snRNPs.

Protein F is known to be one of the common snRNP proteins, together with D, E and G. In the course of the assembly of snRNPs (reviewed by Mattaj [40]), these assemble into an RNA-independent 6S protein hetero-oligomer called "core", prior to attachment to the common structural motif "domain A" of the snRNAs. It was therefore of interest to examine protein F for signs of UV-induced cross-linking to snRNA in the individual snRNPs.

The availability of human autoimmune sera that bind selectively to particle-specific snRNP proteins enabled us to analyse the isolated U1 snRNP labelled *in vivo* with [^{32}P]. By using the anti-(U1)RNP serum P.83, which contains antibodies against the U1 snRNP-specific proteins 70K, A and C, we immunoprecipitated U1 snRNPs from non-irradiated and irradiated mixed snRNPs U1-U6. The U1 snRNPs thus isolated were treated with nucleases and analysed by discontinuous SDS-polyacrylamide gel electrophoresis, as shown in Figure 7A. A control experiment was also performed in order to test the specificity of the isolation of snRNP U1 (Figure 7B). The figure demonstrates clearly that the extraction of U1 was highly specific, and that protein F again is the only non-phosphoprotein labelled covalently by UV irradiation of U1 snRNPs.

It is now generally accepted that the protein core binding to the snRNAs U1, U2, U4 and U5 is always composed of the same set of proteins, as mentioned above. It is therefore likely that the core protein F may bind directly and specifically to the common snRNA area, referred to as the Sm binding site or domain A, or else to a sub-region of this. This is the more probable because an unspecific interaction between the acidic protein F (pI $\leq$ 4; [10]) and the negatively-charged phosphate backbone seems highly unlikely. However, the above supposition will ultimately need to be proved by reconstitution experiments.

Although an unspecific affinity between RNA and protein D has been described [35], no direct interaction between protein D and the snRNA could be detected by UV cross-linking. Nevertheless, an unspecific binding activity of protein D might assist the binding of the protein "core" to the appropriate domain of snRNA.

c) Nucleotide transfer analysis of phosphoprotein 70K

Because of the radioactive labelling of 70K that automatically takes place owing to its phosphorylation *in vivo* (see section (a) above and Figure 3), additional marking of this protein

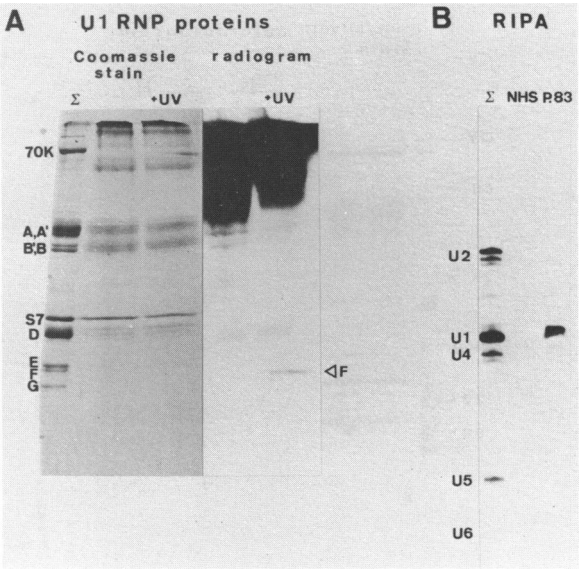

Fig. 7. *UV-induced cross-linking of protein F to U1 snRNA.* U1 RNPs were isolated from 1 x 10^6 cpm *in vivo* ^{32}P-labeled U1-U6 snRNPs irradiated with UV at 254 nm and 18 mJ/mm², or from non-irradiated snRNPs as a control, by using immunoprecipitation with the anti-(U1)RNP serum P.83 (for details, see Materials and Methods and Results (b)).
Panel A: The U1 RNPs were digested with nucleases, and the U1 RNP proteins released were eluted and analysed on an SDS gel. The results of Coomassie blue staining and autoradiography are displayed; the protein F band is seen clearly.
Panel B: In order to check the specificity of this procedure for U1 RNP, the RNA content of control samples, precipitated and not digested, was examined on a 10% polyacrylamide gel containing 7 M urea. Total snRNA is shown for comparison (left); normal human serum fails to bind any of the snRNPs (centre), while the autoimmune serum binds snRNP U1 only (right).

by nucleotide transfer would not be detected by the gel analysis described above. Even if the gel is incubated in alkali in order to dephosphorylate serine residues, the activity of the phosphoserine remaining is more than an order of magnitude greater than that which would be conferred upon protein 70K by nucleotide transfer (cf. Figure 5C).

Therefore, protein 70K of nuclease digested irradiated and non-irradiated snRNPs, respectively, was eluted from the gel and its peptides were analysed by limited proteolysis with chymotrypsin. The resulting peptide pattern, resolved on a 15% SDS-polyacrylamide gel, was examined for additional radioactive fragments.

As the digestion included 4 μg unlabelled and non-irradiated carrier protein 70K, the main fragments of 70K could be detected by staining with Coomassie blue and silver after electrophoresis. The resulting pattern of fragments is shown in Figure 8. Lane 1 shows the polypeptide bands arising from the chymotrypsin used in this assay, in which 2.7 μg of chymotrypsin (ten times more than in the digestion of 70K) was analysed on its own.

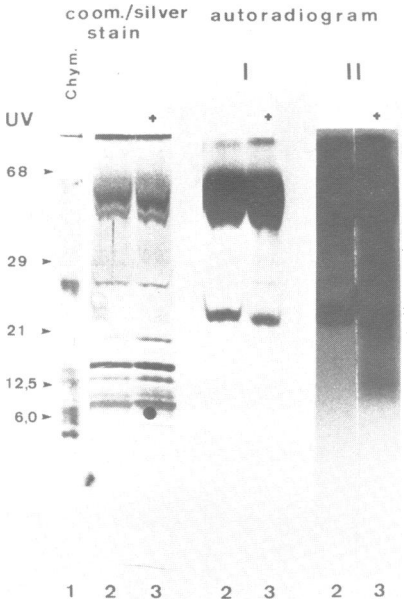

Fig. 8. *Partial proteolysis of protein 70K from non-irradiated and irradiated snRNPs.* U1-U6 snRNPs, labelled in vivo with [^{32}P] and irradiated (254 nm, 18 mJ/mm^2) were digested with nucleases and subjected to electrophoresis on an SDS gel in parallel with a non-irradiated control, as described in Materials and Methods and Results (c). The 70K bands were eluted. 6000 cpm of each were mixed with 4 μg of unlabelled, non-irradiated 70K prepared in the same manner. After removal of SDS, the proteins were digested with 0.27 μg chymotrypsin at 37°C for 30 min [69]. The samples were then analysed by electrophoresis on a 15% polyacrylamide gel. Lane 1: 2.7 μg chymotrypsin incubated alone. Lane 2: non-irradiated 70K. Lane 3: irradiated 70K. Left, Coomassie/silver stain; centre, short autoradiographic exposure; right, long exposure.

In lanes 2 and 3 of the stained gel, the dominant 70K fragments are visible in the range of apparent molecular weight 38-60 kd, and within the lower-MW bands at 16, 13.5, 10.5 and 9.0 kd. Further fragments of lower intensity (lower concentration) can be observed in the range 18-32 kd.

By comparing the stained fragment pattern with the autoradiography from non-irradiated snRNPs (I and II, lane 2) it can be seen that the only phosphorylated fragments detectable after short exposure (I) are in the higher-MW range of 38 to 60 kd, and within one band at 23.5 kd. After longer exposure (II), two additional fragments are observed at 30.5 and 26.3 kd, but none of the relatively abundant low-MW fragments are found to be phosphorylated.

Lane 3 of I and II in Figure 8 shows the pattern of radioactive fragments of protein 70K, isolated from UV-irradiated, nuclease digested [^{32}P]-snRNPs. Although no difference between these and the irradiated controls can be seen after short exposure, prolonged autoradiography reveals a number of additional labelled fragments over the entire size range, down to about 4 kd.

Since intramolecular modification of the protein in response to the mild UV irradiation at 254 nm is highly improbable (see Introduction), and since the protein has been isolated by gel electrophoresis, so that unspecific degradation products are excluded, the most likely explanation for the additional radioactive labelling of 70K fragments is cross-linking to attached oligonucleotides. This is supported by the low intensity of radioactivity of these fragments, compared with the phosphorylation signal (cf. Figure 4 - 6), and by the fact that no phosphorylated proteolytic fragments smaller than 23.5 kd could be detected in the case of the non-irradiated control.

We conclude that, since protein 70K is a U1-specific protein, it is associated directly with the U1 snRNA.

d) Demonstration of the existence of a 70K-U1 RNA complex

The direct association between U1 RNA and protein 70K could be confirmed using a different approach. A protein kinase activity responsible for the phosphorylation of protein 70K co-chromatographs with snRNPs U1-U6 during anti-m_3G chromatographic isolation from HeLa nuclear extracts, as well as with U1 snRNPs in the course of isolation from U1-U6 RNPs by high-pressure liquid chromatography on a "mono Q" ion-exchange column [A.W., P. Bringmann, T. Patschinsky and R.L., manuscript in preparation].

A sample of pure U1 was prepared in this manner. Incubation of purified U1 RNPs with $\gamma[^{32}P]$-ATP, after dialysis against TMN buffer leads to radioactive phosphorylation of the protein 70K *in vitro*. The specificities of the reactions *in vivo* and *in vitro* have been shown to be identical [A.W. et al., cit. ib.]. This provides a possibility for labelling 70K alone within native U1 snRNPs.

Non-irradiated and UV-irradiated U1 snRNPs, labelled specifically by this method, were analysed on an 8-15% polyacrylamide gradient gel containing 0.1% SDS (with focussing on a 5% polyacrylamide stacking gel). Both snRNP proteins and snRNAs migrate in well separated and focussed bands in such a polyacrylamide gel (data not shown). The presumed UV-induced covalent 70K-U1 RNA complex should migrate significantly more slowly than the free phosphoprotein 70K. In a further control, a sample of phosphorylated and irradiated U1 snRNPs was treated with RNase T1 before electrophoresis.

Figure 9 demonstrates that a radioactively labelled band, migrating more slowly than protein 70K, can be detected after UV irradiation of the U1 snRNPs. Incubation with RNase T1 makes this band disappear, which confirms its identity as a complex of protein 70K and U1 snRNA.

As the formation of protein-protein cross-links by irradiation at 254 nm is extremely rare, the relatively high efficiency of cross-linking (about 5%) at the low dose (14 mJ/mm^2) excludes the involvement of any bridging protein. The U1-specific protein 70K therefore is associated directly with the U1 RNA in native U1 snRNPs. Recently, it has been

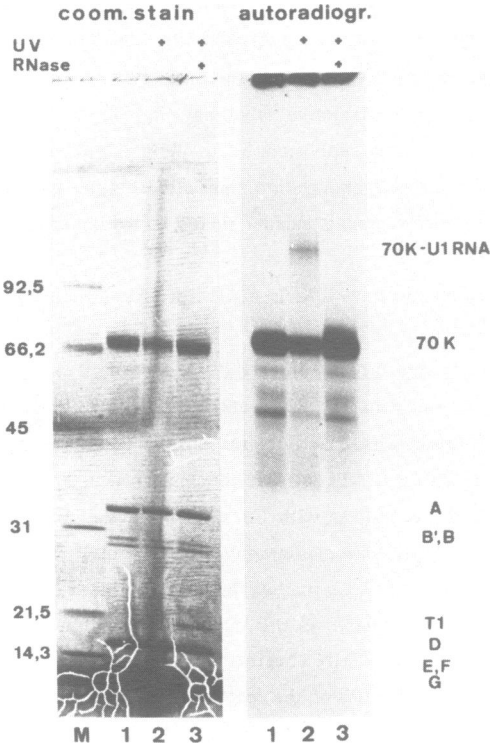

Fig. 9. *UV-induced cross-linking of protein 70K to U1 snRNA.* U1-U6 snRNPs were prepared by anti-m_3G affinity chromatography. Pure U1 snRNPs, containing all the common and all the U1 RNP-specific proteins, were isolated from the U1-U6 mixture on a "mono Q" exchange column. The protein 70K kinase activity co-chromatographs with this U1 fraction. Aliquots of this fraction, each containing 6 μg U1 snRNP, were incubated with $\gamma[^{32}P]$-ATP for 1 h at 37 °C; this resulted in the specific labelling of phosphoprotein 70K. The samples were analysed directly (lane 1), after irradiation with 14 mJ/mm² at 254 nm (lane 2), or after irradiation and subsequent digestion with RNase T1 (lane 3). The electrophoresis was carried out on an 8-15% SDS gradient gel as described in Materials and Methods. The positions of MW markers (M) and of the snRNP proteins are given.

demonstrated that the binding of protein 70K to the U1 RNP requires the presence of the stem and loop of U1 RNA closest to its 5' end, as could be shown by immunoprecipitation with antibodies specific against protein 70K [49,70]. It thus seems likely that direct binding of U1 snRNA to the 5' stem and loop of U1 snRNA is an interaction necessary for the binding of protein 70K to the U1 RNP.

ACKNOWLEDGEMENTS

We would like to acknowledge Gabriele Gutschmidt for excellent technical assistance, Rosita Haupt for culturing of cells and Hannelore Markert for typing the manuscript. We

thank Dr. Montserrat Bach for providing a Mono Q-purified U1 snRNP preparation. The work was supported by the Deutsche Forschungsgemeinschaft (Lu294/3-1). A.W. was supported by a fellowship from the Friedrich-Ebert-Stiftung.

*To whom correspondence should be addressed at: Institut für Molekularbiologie und Tumorforschung, Philipps-Universität, Emil-Mannkopff-Strasse 2, D-3550 Marburg, FRG

REFERENCES

1. Reddy, R. and Busch, H. (1983) Progr. Nucl. Acid Res. Mol. Biol. **30**, 127-162.
2. Brunel, C., Sri-Widada, J. and Jeanteur,. P. (1985) Progr. Molec. Subcell. Biol. **9**, 1-52.
3. Reddy, R. and Busch, H. (1988) In: Structure and function of major and minor small nuclear ribonucleoprotein particles. Birnstiel, M.L. (ed.), Springer-Verlag, Berlin/New York.
4. Bringmann, P., Appel, B., Rinke, J., Reuter, R., Theissen, H. and Lührmann, R. (1984) EMBO J. **3**, 1357-1363.
5. Hashimoto, C. and Steitz, J.A., (1984) Nucl. Acids Res. **12**, 3283-3293.
6. Howard, E.F. (1978) Biochemistry **17**, 3228-3236.
7. Lerner, M.R. and Steitz, J.A. (1979) Proc. Natl. Acad. Sci. USA **76**, 5495-5499.
8. Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J.A. (1980) Nature **283**, 220-224.
9. Brunel, C., Sri-Widada, J., Lelay, M.-N., Jeanteur, P. and Liautard, J.-P. (1981) Nucl. Acids Res. **9**, 815-830.
10. Lührmann, R. (1988) In: Structure and function of major and minor small nuclear ribonucleoprotein particles. Birnstiel, M.L. (ed.) Springer-Verlag, Berlin/New York.
11. Hinterberger, M., Pettersson, I. and Steitz, J.A. (1983) J. Biol. Chem. **258**, 2604-2613.
12. Kinlaw, C.S., Robberson, B.L. and Berget, S.M. (1983) J. Biol. Chem. **258**, 7181-7189.
13. Billings, P.B. and Hoch, S.O. (1984) J. Biol. Chem. **259**, 12850-12856.
14. Pettersson, I., Hinterberger, M., Mimori, T., Gottlieb, E. and Steitz, J.A. (1984) J. Biol. Chem. **259**, 5907-5914.
15. Lelay-Taha, M.-N., Reveillaud, I., Sri-Widada, J., Brunel, C. and Jeanteur, P. (1986) J. Mol. Biol. **189**, 519-532.
16. Bringmann, P. and Lührmann, R. (1986) EMBO J. **5**, 3509-3516.
17. Mimori, T., Hinterberger, M., Pettersson, I. and Steitz, J.A. (1984) J. Biol. Chem. **259**, 560-565.
18. Habets, W., Hoet, M., Bringmann, P., Lührmann, R. and van Venrooij, W. (1985) EMBO J. **4**, 1545-1550.
19. Maniatis, T. and Reed, R. (1987) Nature **325**, 673-678.
20. Sharp, P.A. (1987) Science **235**, 766-771.
21. Steitz, J.A., Black, D.L., Gerke, V., and Parker, K.A. (1988) In: Structure and function of major and minor small nuclear ribonucleoprotein particles. Birnstiel, M.L. (ed.). Springer-Verlag, Berlin/New York.
22. Krainer, A.R. and Maniatis, T. (1988) In: Frontiers in transcription and splicing. Hames, B.D. and Glover, D.M. (eds.) IRL press, Oxford/Washington D.C., in press.
23. Padgett, R.A., Mount, S.M., Steitz, J.A. and Sharp, P.A. (1983) Cell **35**, 101-107.
24. Krämer, A., Keller, W., Appel, B. and Lührmann, R. (1984) Cell **38**, 299-307.
25. Krainer, A.R. and Maniatis, T. (1985) Cell **42**, 725-736.
26. Black, D.L., Chabot, B. and Steitz, J.A. (1985) Cell **42**, 737-750.
27. Berget, S.M. and Robberson, B.L. (1986) Cell **46**, 691-706.
28. Black, D.L. and Steitz, J.A. (1986) Cell **46**, 697-704.
29. Chabot, B. and Steitz, J.A. (1987) Mol. Cell. Biol. **7**, 281-293.
30. Bindereif, A. and Green, M.R. (1987) EMBO J. **6**, 2415-2424.
31. Konarska, M.M. and Sharp, P.A. (1987) Cell **49**, 763-774.
32. Lamond, A.I., Konarska, M.M., Grabowski, P.J. and Sharp, P.A. (1988) Proc. Natl. Acad. Sci. USA **85**, 411-415.
33. Ruskin, B., Zamore, P.D. and Green, M.R. (1988) Cell **52**, 207-219.

34. Mount, S.M., Pettersson, I., Hinterberger, M., Karmas, A. and Steitz, J.A. (1983) Cell **33**, 509-518.
35. Tazi, J., Alibert, C., Temsamani, J., Reveillaud, I., Cathala, G., Brunel, C. and Jeanteur, P. (1986) Cell **47**, 755-766.
36. Gerke, V. and Steitz, J.A. (1986) Cell **47**, 973-984.
37. Frendeway, D. and Keller, W. (1985) Cell **42**, 355-367.
38. Grabowski, P.J., Seiler, S.R. and Sharp, P.A. (1985) Cell **42**, 345-353.
39. Reed, R., Griffith, J. and Maniatis, T. (1988) Cell **53**, 949-961.
40. Mattaj, I.W. (1988) In: Structure and function of major and minor small nuclear ribonucleoprotein particles. Birnstiel, M.L. (ed.) Springer-Verlag, Berlin/New York.
41. Branlant, C., Krol, A., Ebel, J.P., Lazar, E., Haendler, B. and Jacob, M. (1982) EMBO J. **1**, 1259-1265.
42. Jacob, M., Lazar, E., Haendler, B., Gallinaro, H., Krol, A and Branlant, C. (1984) Biol. Cell **51**, 1-10.
43. Liautard, J.P., Sri-Widada, J., Brunel, C., Jeanteur, P. (1982) J. Mol. Biol. **162**, 623-643.
44. Fisher, D.E., Conner, G.E., Reeves, W.H., Wisniewolski, R. and Blobel, G. (1985) Cell **42**, 751-758.
45. Tan, E.M. (1982) Adv. Immunol. **33**, 167-240.
46. Mattaj, I.W. and DeRobertis, E.M. (1985) Cell **40**, 111-118.
47. Mattaj, I.W., Habets, W. and van Venrooij, W. (1986) EMBO J. **5**, 997-1002.
48. Mattaj, I.W. (1986) Cell **46**, 905-911.
49. Hamm, J., Kazmaier, M. and Mattaj, I.W. (1987) EMBO J. **6**, 3479-3485.
50. Markovitz, A. (1972) Biochim. Biophys. Acta **281**, 522-534.
51. Schoemaker, H.J.P. and Schimmel, P.R. (1975) J. Mol. Biol. **84**, 503-513.
52. Möller, K. and Brimacombe, R. (1975) Mol. Gen. Genet. **141**, 343-355.
53. Brimacombe, R. and Stiege, W. (1985) Biochem. J. **229**, 1-17.
54. Van Eekelen, C., Buijtels, H., Linne, T., Ohlsson, R., Philipson, L. and van Venrooij, W. (1982) Nucl. Acids Res. **10**, 3039-3052.
55. Economidis, I. and Pederson, T. (1983) Proc. Natl. Acad. Sci. USA **80**, 1599-1602.
56. Schweiger, A. and Kostka, G. (1985) Biochim. Biophys. Acta **826**, 87-94.
57. Wagenmakers, A., Reinders, R. and van Venrooij, W. (1980) Eur. J. Biochem. **112**, 323-330.
58. Greenberg, J.R. (1980) Nucl. Acids Res. **8**, 5685-5701.
59. Smith, K.C. (1976) Photochem. Photobiol. Nucl. Acids **2**, 187-218.
60. Martinson, H., Shetlar, M. and McCarthy, B. (1976) Biochemistry **15**, 2002-2007.
61. Bringmann, P., Reuter, R., Rinke, J., Appel, B., Bald, R. and Lührmann, R. (1983a) J. Biol. Chem. **258**, 2745-2747.
62. Bochnig, P., Reuter, R., Bringmann, P. and Lührmann, R. (1987) Eur. J. Biochem. **168**, 461-467.
63. Bringmann, P., Rinke, J., Appel, B., Reuter, R. and Lührmann, R. (1983b) EMBO J. **2**, 1129-1135.
64. Stiege, W., Glotz, C. and Brimacombe, R. (1983) Nucleic Acids Res. **11**, 1687-1706.
65. Laemmli, U.K. (1970) Nature **227**, 680-685.
66. Cooper, J.A. and Hunter, T. (1983) Mol. Cell Biol. **1**, 165-178.
67. Gersten, D.M. and Marchalonis, J.J. (1978) J. Immunol. Meth. **24**, 305-309.
68. Reuter, R., Rothe, S. and Lührmann, R. (1987) Nucleic Acids Res. **15**, 4021-4034.
69. Wooley, J.C., Zuckerberg, L.R. and Chung, S.-Y. (1983) Proc. Natl. Acad. Sci. USA **80**, 5208-5212.
70. Patton, J.R. and Pederson, T. (1988) Proc. Natl. Acad. Sci. USA **85**, 747-751.