

Targeting of U2AF⁶⁵ to Sites of Active Splicing in the Nucleus

Margarida Gama-Carvalho,* Randy D. Krauss,‡ Lijian Chiang,‡ Juan Valcárcel,‡ Michael R. Green,‡ and Maria Carmo-Fonseca*

*Institute of Histology and Embryology, Faculty of Medicine, University of Lisbon, 1699 Lisboa Codex, Portugal; and ‡Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Abstract. U2AF⁶⁵ is an essential splicing factor that promotes binding of U2 small nuclear (sn)RNP at the pre-mRNA branchpoint. Here we describe a novel monoclonal antibody that reacts specifically with U2AF⁶⁵. Using this antibody, we show that U2AF⁶⁵ is diffusely distributed in the nucleoplasm with additional concentration in nuclear speckles, which represent subnuclear compartments enriched in splicing snRNPs and other splicing factors. Furthermore, transient expression assays using epitope-tagged deletion mutants of U2AF⁶⁵ indicate that targeting of the protein to nuclear speckles is not affected by removing either the RNA binding domain, the RS domain, or the region required for interaction with U2AF³⁵. The association of U2AF⁶⁵ with speckles persists during mitosis, when transcription and splicing are downregulated. More-

over, U2AF⁶⁵ is localized to nuclear speckles in early G₁ cells that were treated with transcription inhibitors during mitosis, suggesting that the localization of U2AF⁶⁵ in speckles is independent of the presence of pre-mRNA in the nucleus, which is consistent with the idea that speckles represent storage sites for inactive splicing factors. After adenovirus infection, U2AF⁶⁵ re-distributes from the speckles and is preferentially detected at sites of viral transcription. By combining adenoviral infection with transient expression of deletion mutants, we show a specific requirement of the RS domain for recruitment of U2AF⁶⁵ to sites of active splicing in the nucleus. This suggests that interactions involving the RS region of U2AF⁶⁵ may play an important role in targeting this protein to spliceosomes in vivo.

THE splicing of intronic sequences from pre-mRNA occurs within a multicomponent RNA-protein complex called the spliceosome (for review see Moore et al., 1993). The major subunits of the spliceosome are the U1, U2, U4/U6, and U5 small nuclear (sn)¹ RNPs (for review see Baserga and Steitz, 1993). In addition, spliceosomes contain a group of non-snRNP protein splicing factors, several of which have been purified and cloned (for review see Krämer, 1996). In mammalian cells, the best characterized are U2AF (U2 snRNP auxiliary splicing factor), ASF/SF2 (alternative splicing factor/splicing factor 2), and SC-35 (35-kD spliceosomal component). Sequence comparison revealed that these three factors have a common basic structure that can be divided into two functional

subdomains: a consensus type RNA binding domain and a region of arginine/serine (RS) repeats. The RNA-binding domain consists of one or more RNP consensus motifs (RNP-CS) that are required for high affinity and sequence-specific binding of the proteins to RNA (Burd and Dreyfuss, 1994). The RS motif consists of either an uninterrupted stretch of arginine/serine dipeptides or a more dispersed RS-rich region (for review see Lamm and Lamond, 1993). Interestingly, a single monoclonal antibody reacts with a family of at least six RS-rich splicing proteins, including ASF/SF2 and SC-35 (Zahler et al., 1992). The members of this family are commonly referred to as SR protein splicing factors and are highly conserved between *Drosophila* and humans (Mayeda et al., 1992; Zahler et al., 1992). These proteins have been shown to be required for spliceosome assembly as well as for the first step of the splicing reaction, and more recent evidence indicates that they are also implicated in splice site selection and regulated alternative splicing (Wu and Maniatis, 1993; Kohtz et al., 1994).

U2AF is an essential splicing factor required for binding of U2 snRNP to the pre-mRNA branch point (Ruskin et al., 1988; Zamore and Green, 1989). It is composed of two subunits, U2AF⁶⁵ and U2AF³⁵ (Zamore et al., 1992; Zhang et al., 1992). While the 65-kD subunit alone is sufficient to

Randy D. Krauss's present address is Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118-2394.

Juan Valcárcel's present address is Gene Expression Program, European Molecular Biology Laboratory, D-69117 Heidelberg, Germany.

Please address all correspondence to Dr. Maria Carmo-Fonseca, Institute of Histology and Embryology, Faculty of Medicine, University of Lisbon, 1699 Lisboa Codex, Portugal. Tel.: (351) 1-7934340; Fax: (351) 1-7951780.

1. *Abbreviations used in this paper:* CS, consensus sequence; RS, arginine/serine; sn, small nuclear.

reconstitute the *in vitro* splicing activity of nuclear extracts that have been depleted of U2AF by chromatography on poly (U) Sepharose (Zamore and Green, 1991; Zamore et al., 1992; Valcárcel et al., 1993, 1996), a requirement for U2AF⁶⁵ has been documented genetically (Rudner et al., 1996) and biochemically using immunodepleted extracts (Zuo and Maniatis, 1996). The RNA binding domain of U2AF⁶⁵ contains three RNP consensus sequence motifs that specifically bind to the polypyrimidine tract adjacent to the 3' splice site (Zamore et al., 1992; Valcárcel et al., 1993; Singh et al., 1995). The RS domain is located at the NH₂-terminus of the protein and promotes the annealing of U2 snRNA to the pre-mRNA branchpoint (Valcárcel et al., 1996).

Although much is known about the biochemical details of splicing *in vitro*, the organization of RNA processing in the cell nucleus is only starting to be understood. Localization studies have shown that proteins involved in pre-mRNA maturation tend to be heterogeneously distributed in the nucleus, suggesting that the processing reactions might be compartmentalized *in vivo* (Carter et al., 1993). Early electron microscopic studies have clearly established that RNA-protein complexes in the nucleus are localized to distinct types of structures, namely the nucleolus, the perichromatin fibrils, the clusters of interchromatin granules, and the coiled body (for review see Monneron and Bernhard, 1969). Electron microscopic autoradiography has further demonstrated that extranucleolar RNA synthesis occurs in association with perichromatin fibrils, and the more recent techniques of immunoelectron microscopy have shown that several components of the pre-mRNA splicing machinery are associated with perichromatin fibrils (for review see Fakan, 1994). Thus, it is likely that these fibrils represent nascent transcripts with associated spliceosomes. At the light microscopic level it is not possible to resolve perichromatin fibrils, and when immunofluorescence is performed using antibodies that label perichromatin fibrils (for example, antibodies to snRNP or heterogeneous nuclear [hn]RNP proteins), the staining is diffuse in the nucleus, excluding the nucleolus. As perichromatin fibrils are present throughout the nucleoplasm, it is conceivable that the diffuse fluorescent signal represents labeling associated with these fibrils. However, since sn- and hnRNPs are very abundant nuclear components (Rosbash and Singer, 1993; Kiledjian et al., 1994), it is also possible that a pool of excess proteins contributes to the diffuse labeling pattern observed.

In addition to the widespread nucleoplasmic distribution, fluorescence confocal microscopy has revealed that several constituents of the spliceosome appear concentrated in nuclear "speckles" or "foci," and at the electron microscopic level these sites were shown to correspond to clusters of interchromatin granules and coiled bodies, respectively (for review see Spector, 1993). Studies from several laboratories indicate that each of the spliceosomal snRNPs is present widespread in distribution in the nucleoplasm, with an additional concentration in both clusters of interchromatin granules and coiled bodies (for review see Bohmann et al., 1995a), whereas SR protein splicing factors are predominantly detected in speckles (i.e., clusters of interchromatin granules; Fu and Maniatis, 1990; Spector et al., 1991). In contrast, U2AF was described as

diffusely distributed in the nucleoplasm with additional concentration in coiled bodies (Carmo-Fonseca et al., 1991; Zamore and Green, 1991; Zhang et al., 1992). However, more recent evidence indicates that the RS domain of the *Drosophila* splicing protein Tra contains a sequence that can function both as a nuclear localization signal and a speckle localization signal (Hedley et al., 1995). As a similar amino acid sequence motif is found in the RS region of U2AF⁶⁵, this protein should also localize to nuclear speckles.

In this report we describe a novel monoclonal antibody that specifically reacts with U2AF⁶⁵, and we show that this protein colocalizes with splicing snRNPs and SR protein splicing factors in nuclear speckles. We also present evidence that the association of U2AF⁶⁵ with the nuclear speckles is unrelated to splicing activity, consistent with the idea that these structures represent a subnuclear compartment dedicated to storage or preassembly of the splicing machinery. Finally, the data indicate that the RS domain of U2AF⁶⁵ is required for recruitment to active splicing sites *in vivo*.

Materials and Methods

Production of mAbs

Eight female BALB/c mice were immunized with purified recombinant U2AF⁶⁵ expressed in *Escherichia coli*. The protein was diluted with PBS to a concentration of 0.2 µg/ml, emulsified with Freund's adjuvant (Difco Laboratories, Detroit, Michigan), and injected intraperitoneally. Mice were boosted twice at 3 wk intervals, and test bleeds were taken. The sera were screened by ELISA and immunoblotting using recombinant U2AF⁶⁵ as antigen. The sera from all animals tested positive in these assays but varied significantly in antibody titer. The spleen cells from the two mice with higher antiserum titer were fused with Ag8.653 myeloma cells, and hybrids were selected as described by Harlow and Lane (1988). After 10 d, hybridoma supernatants were tested for reactivity with recombinant U2AF⁶⁵ by ELISA. Of 480 fusion wells, 3 tested positive and 2 (MC2 and MC3) were successfully cloned by limiting dilution on microtiter plates. The two hybridoma cell lines were used to induce a peritoneal tumor in pristane primed adult female mice, and ascitic fluid was collected (Harlow and Lane, 1988). The antibody subtypes were determined using a kit from Boehringer Mannheim GmbH (Mannheim, Germany).

Immunoblotting, Immunodepletion, and Complementation Assays

Total cell protein extracts were prepared by scraping the cells with a rubber policeman into SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) with 200 U/ml benzonase (Sigma Chemical Co., St. Louis, MO), incubating for 15 min at room temperature (to digest DNA), and then boiling for 5 min. The SR protein extract was prepared as described by Zahler et al. (1992). *Drosophila* and *Xenopus* protein extracts were prepared from SL2 and MCM cells, respectively. Proteins were separated on either 8 or 12% acrylamide gels and transferred to nitrocellulose membranes using a semi-dry electrotransfer apparatus (BioRad Laboratories, Richmond, California). The membranes were blocked and washed with 2% nonfat milk powder in PBS. The blots were incubated for 1 h with hybridoma supernatant diluted in washing buffer, washed, and incubated for 1 h with secondary antibody conjugated to alkaline phosphatase (BioRad Laboratories).

Immunodepletion of nuclear extracts was performed as follows: 100 µl of protein A-agarose beads (Sigma Chemical Co.) were washed twice with 500 µl of buffer D (20 mM Hepes, pH 8.0, 0.2 mM EDTA, 20% glycerol, 0.1M KCl, 1 mM DTT, 0.05% NP-40) on ice and incubated with 200 µl of MC3 supernatant for 90 min at 4°C. After two washes in the same buffer, the beads were added to 100 µl of nuclear extract (Dignam et al., 1983) and incubated for 3 h at 4°C. The depleted extract was collected by

centrifugation. For the complementation assays, splicing reactions were performed with 10 fmols of an adeno major late promoter-derived pre-mRNA substrate, 22% HeLa nuclear extract (either complete or MC3-depleted), 3.3% polyvinyl alcohol, 1.67 mM MgCl₂, 25 mM KCl, 1 mM ATP, 22 mM creatine phosphate, in the presence or absence of 12.5 ng/μl of recombinant purified GST-U2AF⁶⁵ protein, for 2 h at 30°C. After proteinase K, phenol extraction, and precipitation, the products of the splicing reaction were analyzed by PAGE on a 13% gel.

Cell Culture, Drug Treatment, and Viral Infection

HeLa cells were grown as monolayers in Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum and maintained mycoplasma free. Mitotic cells were isolated by the shake-off method, allowed to sediment by gravity for 1 h on ice, plated on glass coverslips, and further incubated at 37°C for 50–60 min to obtain a population enriched in late telophase/early G₁ cells. For inhibition of transcriptional activity, cells were treated for 1 to 2 h with either 5 μg/ml actinomycin D (Sigma Chemical Co.) or 75 μM DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Sigma Chemical Co.). Reversal of the DRB transcription block was made by washing the cells three times with fresh medium and allowing them to recover for 1 h at 37°C. Infection of HeLa cells with adenovirus type 2 (Ad2) was performed as described (Pombo et al., 1994).

Transient Transfection Assays

The following primers were prepared. T8: 5'-CCGGTACCGACACCATGTACGACTACGTCCTGATTAG CAATGTCGGACTTCGAC-3'. T9: 5'-GGACGCGTCTACCAGAAGTCCCGGCGGTG-3'. T10: 5'-GGACGCGTCTACTGTGGGCAGAGTCGGG-3'. NLR1: 5'-CTCGTGGAGGGGGAACGAATCAG-3'. RK36: 5'-CTCTACGTGGGCAACATCCC-3'. RK38: 5'-CTGTTCA TCGGGGGCTTACCC-3'. Primer T8 was designed to encode a 10 amino acid hemagglutinin (HA) epitope tag to distinguish the transiently expressed protein from the endogenous protein. Templates for PCR were as described by Zamore et al. (1992). HA-tagged U2AF⁶⁵WT and U2AF⁶⁵ΔRS cDNAs were prepared using primers T8 and T9. U2AF⁶⁵ARNP2,3 was prepared using primers T8 and T10. U2AF⁶⁵Δ84-150 and U2AF⁶⁵Δ84-260 were prepared using pECE-U2AF⁶⁵T (Zamore et al., 1992) and primers NLR1/RK36 and NLR1/RK38, respectively. PCR was carried out under the following conditions: 94°C for 30 s, 64°C for 30 s, and 72°C for 3 min. Upon completion of the 30 cycles, an additional elongation step was carried out for 7 min at 72°C. The products and the expression vector CMV5 (see Andersson et al., 1989; kindly provided by Dr. Maria Zapp, University of Massachusetts Medical Center, Worcester, MA) were cut with KpnI and MluI for ligation. The ligated product was transformed into DH5α. DNA sequence analysis was performed to determine if the clones contained the correct deletions. DNA constructs were transiently transfected into cells, and Western blot analysis was performed using anti-HA antibodies to determine if the constructs were being expressed (data not shown). Transient transfection of HeLa cells was performed using Lipofectin (GIBCO BRL, Gaithersburg, MD), according to the manufacturer's instructions. Approximately 2 μg of DNA was used per assay. The cells were analyzed by immunofluorescence at 24 to 48 h after transfection.

Immunofluorescence

For indirect immunofluorescence the cells were grown on 10 × 10-mm glass coverslips and harvested at 60–80% confluency. Coverslips with attached cells were washed twice in PBS and treated according to the following alternative protocols: (a) immediate fixation with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 10 min at room temperature and subsequent permeabilization with 0.5% Triton X-100 in PBS for 15 min at room temperature; (b) permeabilization with 0.5% Triton X-100 in CSK buffer (Fey et al., 1986) containing 0.1 mM PMSF for 1 min on ice and subsequent fixation with 3.7% formaldehyde in CSK for 10 min at room temperature. After fixation and permeabilization, the cells were rinsed in PBS containing 0.05% Tween 20 (PBS-T), incubated for 1 h with primary antibodies diluted in PBS-T, washed, and incubated for 30 min with the appropriate secondary antibodies conjugated to either fluorescein or Texas red (Dianova GmbH, Hamburg, Germany; Vector Laboratories, Peterborough, UK). Finally, the coverslips were mounted in VectaShield (Vector Laboratories) and sealed with nail polish.

In addition to the mAbs directed against U2AF⁶⁵, the following antibodies were used in this study: human autoantiserum C45, specific for Sm

proteins (kindly provided by Professor W. van Venrooij, University of Nijmegen, The Netherlands), mAb 3C5 directed against SR proteins (Turner and Franchi., 1987; Bridge et al., 1995), rabbit polyclonal serum 204.4 directed against the coiled body protein p80-coilin (Bohmann et al., 1995b), rabbit polyclonal serum directed against the adenoviral protein DBP (Linné et al., 1977), mAb 4B10 specific for hnRNP protein A1 (Piñol-Roma et al., 1988), and mAb 12CA5-1 directed against the HA epitope (Berkeley Antibody Company, Richmond, CA). Note that double labeling with the mAbs 3C5 and MC3 was possible because 3C5 is an IgM and MC3 is an IgG. To control the specificity of the secondary antibodies, the cells were incubated with each primary antibody alone and then incubated alternatively with either anti-IgM or anti-IgG conjugates.

In situ hybridization to detect poly A RNA was performed as described (Carmo-Fonseca et al., 1992) using a biotinylated 2'-O-methyl oligoribonucleotide probe (Sproat et al., 1989) containing 20 tandem uridine residues. Detection of adenoviral RNA was performed as described (Pombo et al., 1994).

Fluorescence and Confocal Microscopy

Samples were examined with a microscope (LSM 410; Zeiss, Inc., Oberkochen, Germany). Confocal microscopy was performed using argon ion (488 nm) and HeNe (543 nm) lasers to excite FITC and Texas red fluorescence, respectively. For double labeling experiments, images from the same focal plane were sequentially recorded and superimposed. To obtain a precise alignment of superimposed images the equipment was calibrated using multicolor fluorescent beads (Molecular Probes Inc., Eugene, OR) and a dual-band filter that allows simultaneous visualization of red and green fluorescence. The images were photographed on Fujichrome 100 or Kodak TMax 100 film, using a freeze frame recorder (Polaroid Corp., Cambridge, MA). Alternatively, data files were directly printed on a digital printer (XLS 8300; Kodak Co., Rochester, NY).

Results

Monoclonal Antibody MC3 Specifically Recognizes U2AF⁶⁵

To obtain monoclonal antibodies, hybridomas were derived by fusion of the mouse myeloma cell line Ag8.653 with spleen cells from BALB/c mice immunized with recombinant U2AF⁶⁵. Two clones, MC2 and MC3, tested positive by ELISA using purified recombinant U2AF⁶⁵ as antigen. The antibody secreted by the MC2 clone is of the IgG₁ class, and mAb MC3 is IgG_{2b}. Immunoblot analysis reveals that both mAbs recognize a single ~65-kD band in HeLa protein extracts, that migrates with the same apparent molecular weight as the recombinant U2AF⁶⁵ protein used for immunizations (Fig. 1 A, and data not shown). Since the mAb MC3 reacts with U2AF⁶⁵ with much higher affinity than mAb MC2, it was chosen for further use. Western blot analysis using a nuclear extract enriched in SR proteins (Zahler et al., 1992) shows that mAb MC3 does not crossreact with the SR family of protein splicing factors (Fig. 1 A). The mAb MC3 recognized a ~65-kD protein from *Xenopus* but failed to react with *Drosophila* protein extracts (results not shown).

As U2AF⁶⁵ is an essential splicing factor, we tested mAb MC3 for the ability to deplete splicing activity from nuclear extracts. Western blot analysis reveals that U2AF⁶⁵ is almost completely depleted from nuclear extracts incubated with mAb MC3 bound to protein A-agarose beads (Fig. 1 B; the immunodepletion efficiency ranged between 90 and 99%). The depleted extracts do not support in vitro splicing reactions, but splicing activity can be successfully recovered by the addition of recombinant U2AF⁶⁵ (Fig. 1 C). Although the incubation of nuclear extracts with mAb MC3 results in depletion of both U2AF⁶⁵ and U2AF³⁵ (re-

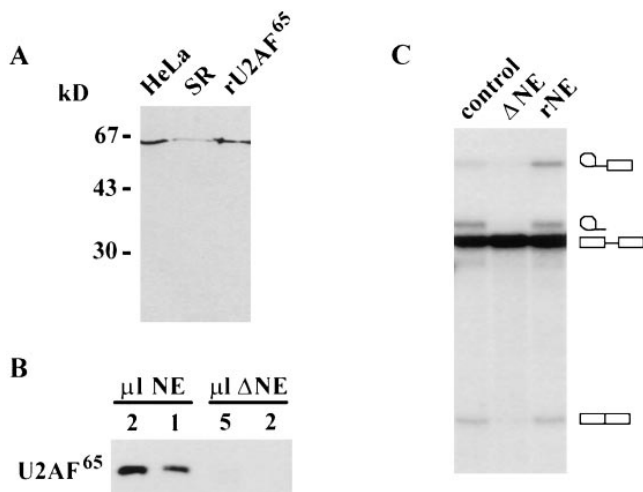


Figure 1. The mAb MC3 reacts specifically with U2AF⁶⁵. (A) Immunoblot analysis was performed using MC3 hybridoma supernatant diluted 1:100. A 12% acrylamide gel was loaded with total protein extract from HeLa cells ($1-4 \times 10^5$ cells/well), SR protein-enriched fraction (10 μg /well), and purified recombinant U2AF⁶⁵ (0.2 μg /well). Molecular weight markers are indicated on the left. (B) Immunoblot analysis of nuclear extracts (1, 2, or 5 μl per lane, as indicated). NE, control nuclear extract; ΔNE , nuclear extract immunodepleted using mAb MC3. (C) In vitro reconstitution/splicing reactions were performed using nuclear extract (control), U2AF⁶⁵-immunodepleted extract (ΔNE), or U2AF⁶⁵-immunodepleted extract plus 12.5 ng/ μl of purified recombinant U2AF⁶⁵ (rNE).

sults not shown), addition of U2AF³⁵ is not required to restore splicing activity, as previously described (Zamore and Green, 1991).

Using a series of GST-U2AF⁶⁵ deletion mutants (Valcárcel et al., 1996), we conclude that the epitope recognized by mAb MC3 maps between amino acids 138 and 161 (Fig. 2).

Subnuclear Localization of U2AF⁶⁵

Indirect immunofluorescence was performed using mAbs MC2 and MC3. Both antibodies produced a diffuse staining of the nucleoplasm excluding the nucleoli, with additional concentration in 20–50 speckles (Fig. 3 A). These results were obtained in HeLa and Hep-2 cells as well as in cryosections from rat brain tissue (data not shown). Similar results were observed when cultured cells were treated with Triton X-100 either before or after fixation with formaldehyde or when the antibody was directly microinjected into the nuclei of living cells (Fig. 3 D). Double labeling experiments were performed using the mAb MC3 and either an anti-Sm antibody, which reacts with all splicing snRNPs (Baserga and Steitz, 1993), mAb 3C5, which recognizes SR protein splicing factors (Bridge et al., 1995) and labels clusters of interchromatin granules by immunoelectron microscopy (Turner and Franchi, 1987), or an anti-coilin antibody to label coiled bodies (Bohmann et al., 1995b). The results show that U2AF⁶⁵ colocalizes with snRNPs and SR proteins in nuclear speckles (Fig. 3, B and C, and data not shown), but labeling of coiled bodies was never detected (Fig. 3, D and E, and data not shown). This

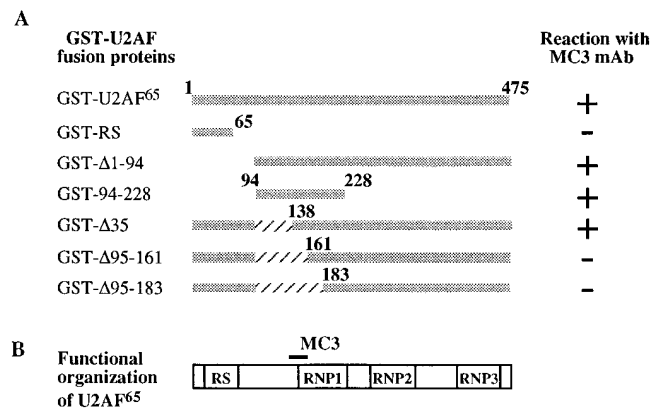


Figure 2. Epitope mapping of U2AF⁶⁵. (A) Diagram showing the reactivity of mAb MC3 with different GST-U2AF⁶⁵ fusion proteins as determined by immunoblot analysis. The grey bars represent the U2AF⁶⁵ fragment fused to the GST protein. Hatched regions represent internal deletions of U2AF⁶⁵. (B) Diagram showing the mapped epitope (amino acids 138–161, bar) in relation to the functional organization of U2AF⁶⁵. The mapped region overlaps with the beginning of the RNA binding domain (amino acids 151–462).

contrasts with the results previously obtained using a purified polyclonal antibody raised against a synthetic peptide of U2AF⁶⁵, which produced a predominantly diffuse nucleoplasmic staining with additional concentration in coiled bodies (Carmo-Fonseca et al., 1991; Zamore and Green, 1991). In this regard it is noteworthy that when the mAb MC3 is used at high dilutions it also produces a predominantly diffuse staining pattern. This argues that the failure to detect speckles previously could have been due to a low titer of the anti-peptide antibody. Furthermore, when cells are treated with actinomycin D for 1–2 h, U2AF⁶⁵ is detected both in enlarged speckles and in perinucleolar patches (Fig. 3 F, arrowheads, and G). As a similar perinucleolar staining was previously observed (Carmo-Fonseca et al., 1991), this suggests that in vivo the antipeptide antibody reacts preferentially with a subfraction of the total U2AF⁶⁵ in the nucleus. In contrast, the reason for the differential recognition of coiled body epitopes in vivo by these two antibodies remains to be elucidated. Clearly, there are peculiar properties of the epitopes recognized by the distinct antibodies, and to solve this discrepancy we have analyzed the distribution of epitope-tagged U2AF⁶⁵ in transiently transfected HeLa cells. The results observed are identical to those revealed by the new monoclonal antibodies (Figs. 3 A and 8 A), allowing us to settle the controversy.

U2AF⁶⁵ Associates with Interchromatin Granules during Mitosis

To investigate whether the presence of U2AF⁶⁵ in nuclear speckles is associated with splicing, we studied the distribution of U2AF⁶⁵ during mitosis, when transcription and hence splicing activities are inhibited (Prescott and Bender, 1962). From metaphase through telophase the mAb MC3 produces a diffuse staining of the cell excluding the chromosomes, with additional concentration in speckled struc-

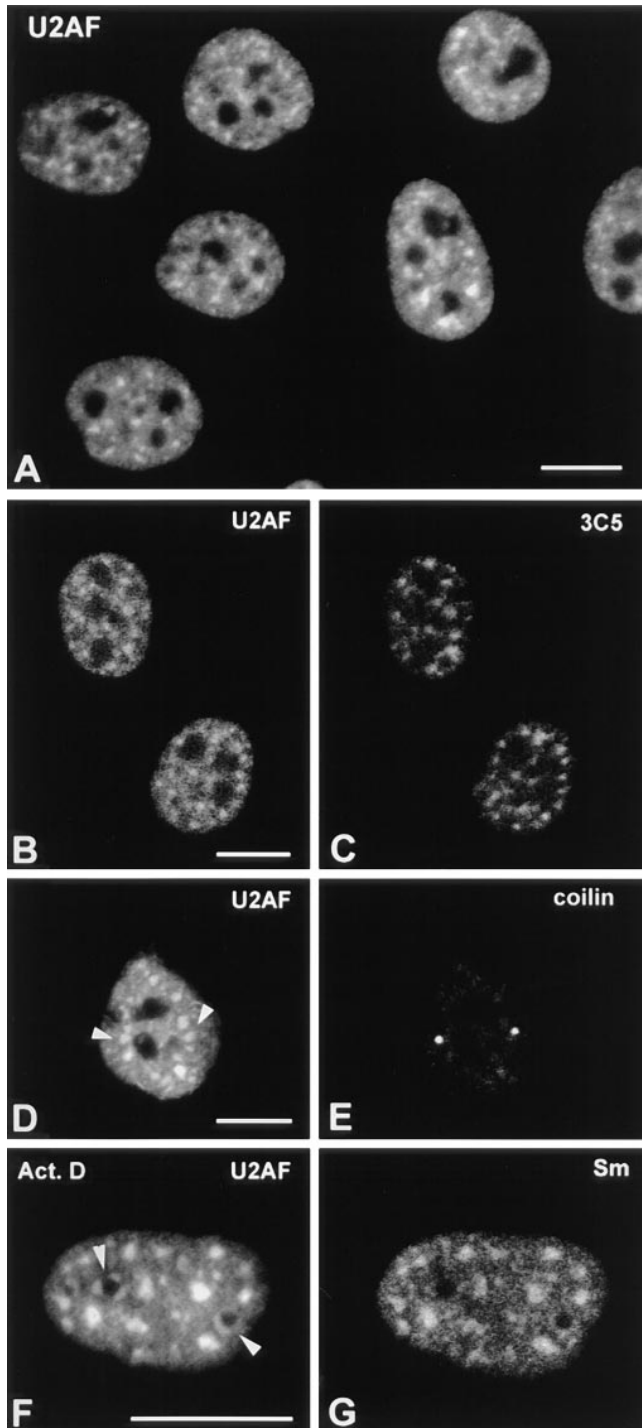


Figure 3. Subnuclear localization of U2AF⁶⁵. (A) HeLa cells were fixed with formaldehyde, permeabilized with Triton X-100, and incubated with mAb MC3 (hybridoma supernatant undiluted). (B and C) HeLa cells were permeabilized with Triton X-100 before fixation. Double labeling was then performed using mAb MC3 (B) and mAb 3C5 (C). (D and E) Living HeLa cells were microinjected in the nucleus with MC3 ascitic fluid and further incubated at 37°C for 30 min. Then, the cells were fixed in formaldehyde, permeabilized with Triton X-100, and sequentially incubated with anti-coilin rabbit polyclonal antibody, anti-rabbit IgG coupled to Texas red (E), and anti-mouse IgG coupled to FITC (D). In D, arrowheads indicate the position of coiled bodies as observed on overlays of both fluorescence signals. The cells depicted in panels F and G were incubated with 5 μg/ml actinomycin D for 2 h, permeabilized with Triton X-100, fixed in formaldehyde, and double labeled with MC3 (F) and anti-Sm antibodies (G). Arrowheads in F indicate staining of perinucleolar structures. Bars, 10 μm.

tures (Fig. 4, A–C). Double labeling experiments using the mAb MC3 and anti-Sm antibody (Fig. 4, D–L) or the mAb 3C5 (data not shown) indicate that U2AF⁶⁵ colocalizes with snRNPs and SR proteins in mitotic speckles that correspond to mitotic clusters of interchromatin granules (MIGs; Ferreira et al., 1994). In contrast, a colocalization of U2AF⁶⁵ with mitotic coiled bodies was never detected. The association of U2AF⁶⁵ with mitotic speckles is most prominent during telophase, when the number and size of MIGs is higher (Ferreira et al., 1994). In late telophase/early G₁ cells, U2AF⁶⁵ and snRNPs are predominantly detected within the nuclei of daughter cells, whereas the mAb 3C5 labels large speckles that persist in the cytoplasm (Fig. 4, M–O). This suggests that at the end of mitosis, U2AF⁶⁵ and snRNPs leave the MIGs and are rapidly transported into the daughter cell nuclei, whereas SR proteins remain associated with MIGs in the cytoplasm for a longer period of time.

U2AF⁶⁵ Associates with Speckles in the Absence of Nuclear poly (A) RNA Synthesis

Having established that U2AF⁶⁵ associates with interchromatin granules during mitosis when there is no transcriptional activity, we next asked whether U2AF⁶⁵ can be targeted to nuclear speckles in the absence of poly (A) RNA synthesis. Mitotic HeLa cells were treated with either 5 μg/ml actinomycin D or 75 μM DRB for 1 h to inhibit transcriptional activity, and immunofluorescence was performed using the mAb MC3. In all early G₁ cells observed, the staining was concentrated in nuclear speckles, and double labeling experiments using anti-Sm antibodies indicate that inhibition of transcription does not affect the targeting of either U2AF⁶⁵ or snRNPs to nuclear speckles (Fig. 5, A–C). A parallel labeling of the cells with a mAb for hnRNP A1 confirmed the efficiency of the transcription block, since transport of this protein into the nucleus at the end of mitosis is transcription dependent (Piñol-Roma and Dreyfuss, 1991). In addition, in situ hybridization experiments using a poly (U) oligonucleotide probe fail to detect any poly (A) RNA signal in the nuclei of early G₁ cells treated with transcription inhibitors during mitosis (Fig. 5, D–F). Taken together, these data suggest that the localization of U2AF⁶⁵ to nuclear speckles is unrelated to splicing activity.

The RS Region of U2AF⁶⁵ Is Not Required for Targeting to Nuclear Speckles

Recently, Hedley et al. (1995) identified an amino acid sequence within the RS region of the *Drosophila* protein Tra that is sufficient to target an heterologous protein to nuclear speckles. Since a similar sequence motif (i.e., a stretch of basic amino acids followed by RS dipeptides and a putative bipartite NLS) is present in the RS region of U2AF⁶⁵ (Fig. 6 A), we decided to study the role of the U2AF⁶⁵ RS

depicted in panels F and G were incubated with 5 μg/ml actinomycin D for 2 h, permeabilized with Triton X-100, fixed in formaldehyde, and double labeled with MC3 (F) and anti-Sm antibodies (G). Arrowheads in F indicate staining of perinucleolar structures. Bars, 10 μm.

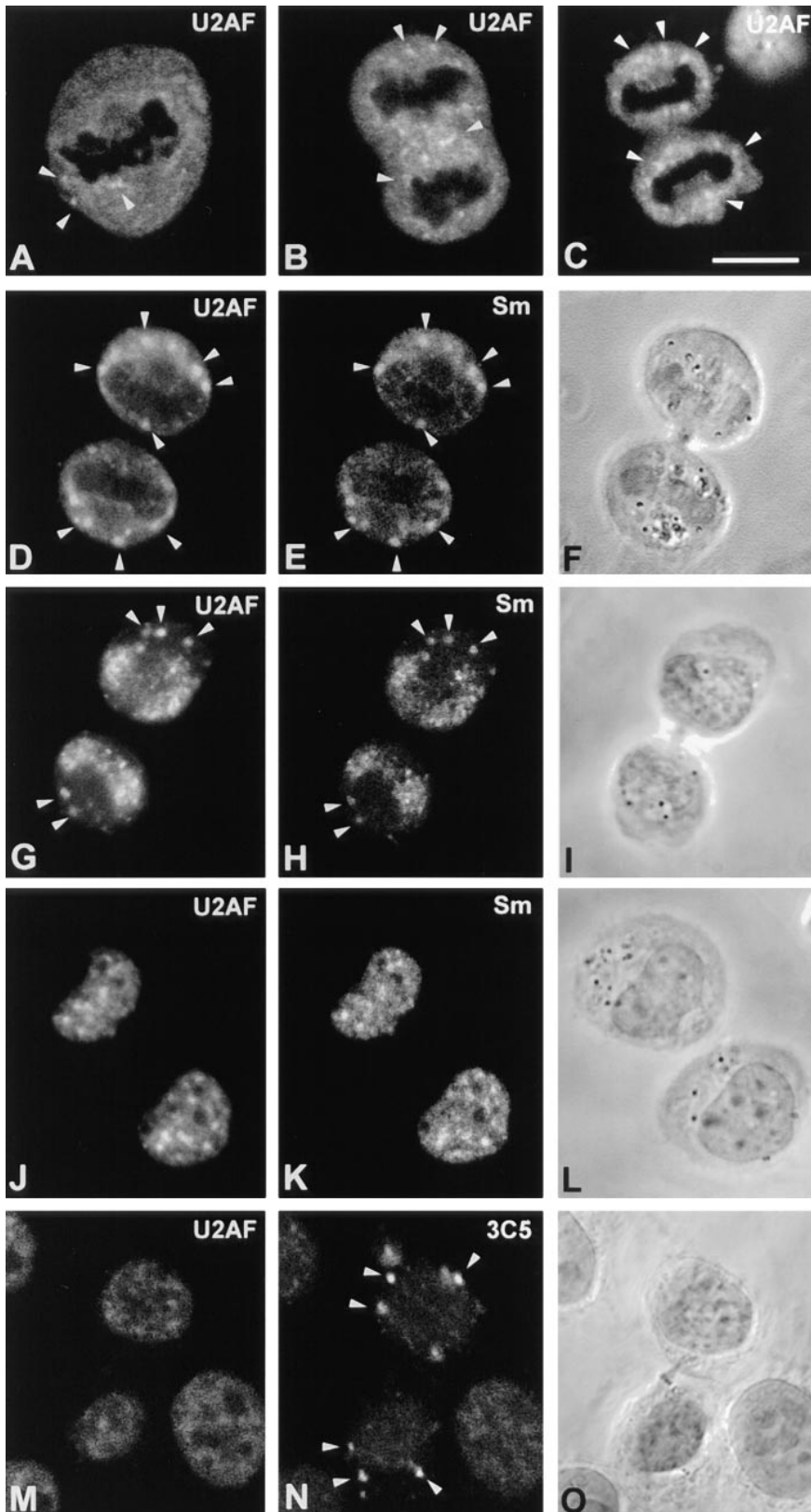


Figure 4. Intracellular distribution of U2AF⁶⁵ during mitosis. HeLa cells were fixed with formaldehyde, permeabilized with Triton X-100, and incubated with mAb MC3 (A–C) or double-labeled with mAb MC3 and anti-Sm antibody (D–L). From metaphase to telophase, the staining produced by mAb MC3 is diffuse in the cytoplasm, excluding the chromosomes, with additional concentration in speckles (A–C, arrowheads). During the early stages of telophase, both U2AF and snRNPs colocalize in mitotic speckles and are excluded from the newly forming nucleus (D–F). As telophase proceeds, U2AF and snRNPs are simultaneously detected in mitotic speckles (arrowheads) and within the daughter cell nuclei (G–I), whereas in early G₁ cells, U2AF and snRNPs are exclusively detected in the nucleus (J–L). For double labeling experiments using mAbs MC3 and 3C5, the cells were extracted with Triton X-100 before formaldehyde fixation (M–O). These experiments show that in late telophase cells, SR proteins persist associated with mitotic speckles (N, arrowheads), while U2AF is predominantly localized to the nucleus (M). Inspection of cells at earlier stages of telophase confirm the presence of U2AF in mitotic speckles in these preparations, indicating that this lack of colocalization between U2AF and SR proteins is not an artifact induced by the preextraction procedure. Bar, 10 μ m.

region in subnuclear localization. Transient transfection assays were performed on HeLa cells using the pCMV5 expression vector (Andersson et al., 1989). The vector contained a 10 amino acid influenza HA epitope tag cloned in frame in front of the cDNA coding for U2AF⁶⁵. Four

deletion mutants were analyzed: U2AF⁶⁵ Δ RS-T has a deletion of amino acids 23–65, covering all the RS domain; U2AF⁶⁵ Δ RNP2,3-T has a deletion of amino acids 260–475, spanning the RNA binding regions RNP2 and RNP3; U2AF⁶⁵ Δ 84–150-T has a deletion of amino acids 84–150,

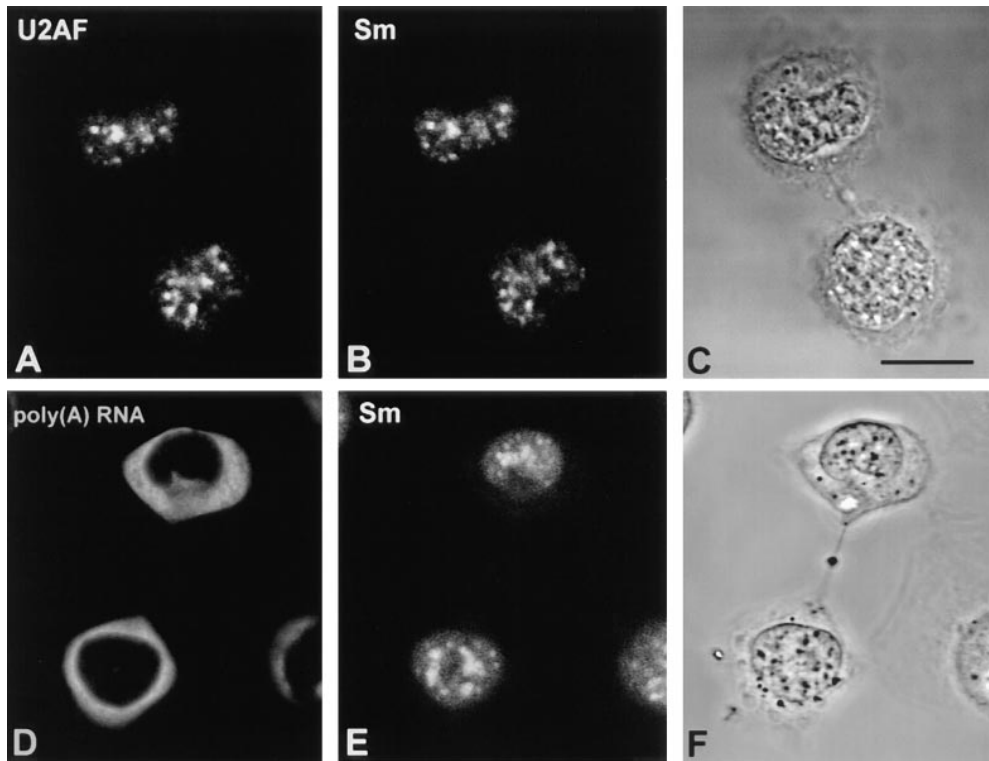


Figure 5. U2AF⁶⁵ associates with nuclear speckles in the absence of RNA synthesis. Mitotic HeLa cells were treated with 5 μg/ml actinomycin D for 1 h, and then double immunofluorescence was performed using mAb MC3 and anti-Sm antibody. The labeling produced by both antibodies is clearly concentrated in nuclear speckles (A–C). A similar experiment was performed with the transcription inhibitor DRB; then, the cells were hybridized in situ with a poly (U) riboprobe and immunolabeled with anti-Sm antibody. No poly (A) RNA is detected in the nucleus, while snRNPs are clearly concentrated in nuclear speckles (D–F). Bar, 10 μm.

covering the region of interaction with U2AF³⁵; and U2AF⁶⁵Δ84-260-T has a deletion of amino acids 84–260 spanning both the region of interaction with U2AF³⁵ and the RNP1 domain (Fig. 6 B).

The mAb 12CA5, which is specific for the HA tag, produces a faint background staining on untransfected HeLa cells or on cells transfected with the vector alone (data not shown). When cells are transfected with either epitope-tagged recombinant U2AF⁶⁵ (U2AF⁶⁵WT-T) or either of the deletion mutants, the mAb 12CA5 displays an intense staining of the nucleus (Fig. 7). At higher magnification the nuclear staining pattern is clearly speckled, similar to that observed with mAb MC3 (Figs. 3 A and 8 A). Double labeling of the transfected cells with mAb 12CA5 and anti-Sm antibodies show that U2AF⁶⁵WT-T colocalizes with splicing snRNPs in nuclear speckles but not in coiled bodies (Fig. 8, A and E, and data not shown). Interestingly, the deletion mutants U2AF⁶⁵ΔRS-T, U2AF⁶⁵ΔRNP2,3-T, U2AF⁶⁵Δ84-150-T, and U2AF⁶⁵Δ84-260-T retained the ability to localize to nuclear speckles (Fig. 8, B and F, C and G, D and H, and data not shown). Thus, neither of these domains of U2AF⁶⁵ on its own is essential to target the protein to nuclear speckles.

The RS Region of U2AF⁶⁵ Is Involved in Recruitment to Splicing Sites

Since the presence of U2AF⁶⁵ in nuclear speckles appears to be unrelated to splicing activity, we tried to visualize the recruitment of this protein to active splicing sites within the nucleus. For this we have made use of adenovirus infection, because this virus subverts the normal nuclear organization by recruiting the host transcription and processing machinery to the sites of viral mRNA synthesis

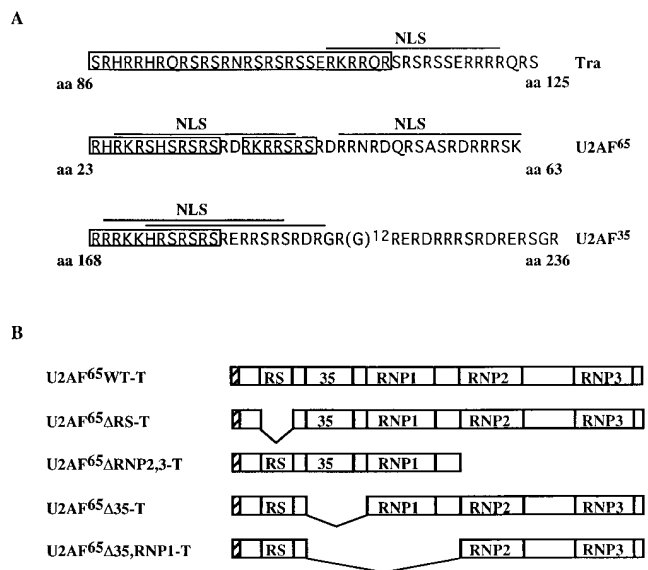


Figure 6. Both U2AF⁶⁵ and U2AF³⁵ contain putative “speckle localization signals.” (A) Diagram showing the putative speckle localization signal of the *Drosophila melanogaster* splicing factor Tra, identified by Hedley et al. (1995) and similar sequence motifs present in U2AF⁶⁵ and U2AF³⁵ that may represent potential nuclear and subnuclear localization signals. Shown is the amino acid sequence of the SR domain of both proteins. Sequences containing three or four basic residues directly adjacent to RS dipeptides are boxed. Putative nucleoplasm-like NLSs are indicated. (B) A diagram of the constructs used for transfection is shown: ▨, HA-tag; 35, binding region for U2AF³⁵.

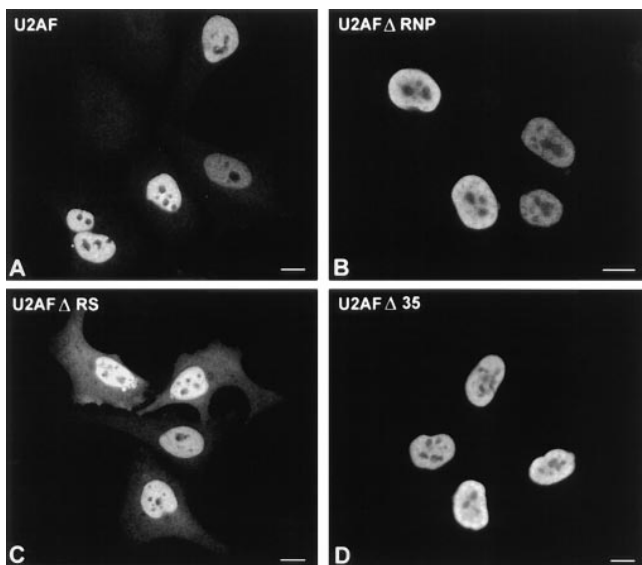


Figure 7. Epitope-tagged recombinant U2AF is targeted to the nucleus. HeLa cells were transfected with the pCMV5 expression vector containing a 10-amino acid influenza HA epitope tag cloned in frame in front of the cDNA coding for either U2AF⁶⁵ (A), U2AF⁶⁵ΔRNP2,3 (B), U2AF⁶⁵ΔRS (C), or U2AF⁶⁵Δ84-150 (D). At 24 to 36 h after transfection the cells were fixed with formaldehyde and permeabilized with Triton X-100, and the ectopically expressed protein was detected using mAb 12CA5 directed against the HA tag. Bars, 10 μm.

(Jiménez-García and Spector, 1993; Pombo et al., 1994). When adenovirus-infected HeLa cells are probed with mAb MC3, the nuclear staining pattern changes significantly depending on the stage of infection. During the early phase of infection (i.e., before the onset of major viral DNA replication, which occurs at ~8 h after infection), U2AF⁶⁵ is detected in speckles, similar to that observed in noninfected cells (data not shown). After the onset of viral replication the normal nuclear architecture is grossly changed, and U2AF⁶⁵ is no longer observed in speckles. Rather, in cells infected for 14–18 h the U2AF⁶⁵ staining is predominantly detected in ring-like structures that surround the sites of viral DNA labeled with an antibody directed against the adenovirus DNA binding protein, DBP. As we had previously shown that adenovirus induces a redistribution of splicing snRNPs into ring-like structures that colocalize with the sites of viral transcription (Pombo et al., 1994), double labeling experiments were performed using mAb MC3 and anti-Sm antibodies. The results indicate that U2AF⁶⁵ colocalizes with snRNPs in the ring-like structures (Fig. 9, A–D), and similar results are observed on cells that were sequentially transfected with U2AF⁶⁵WT-T, infected with adenovirus, and probed with anti-tag antibody (Fig. 9, E and F). Thus, we conclude that the ring structures labeled by MC3 and anti-tag antibody correspond to U2AF⁶⁵ at sites of active splicing. At later stages of infection (20–24 h after infection), the ring-like structures become less prominent, and U2AF⁶⁵ is predom-

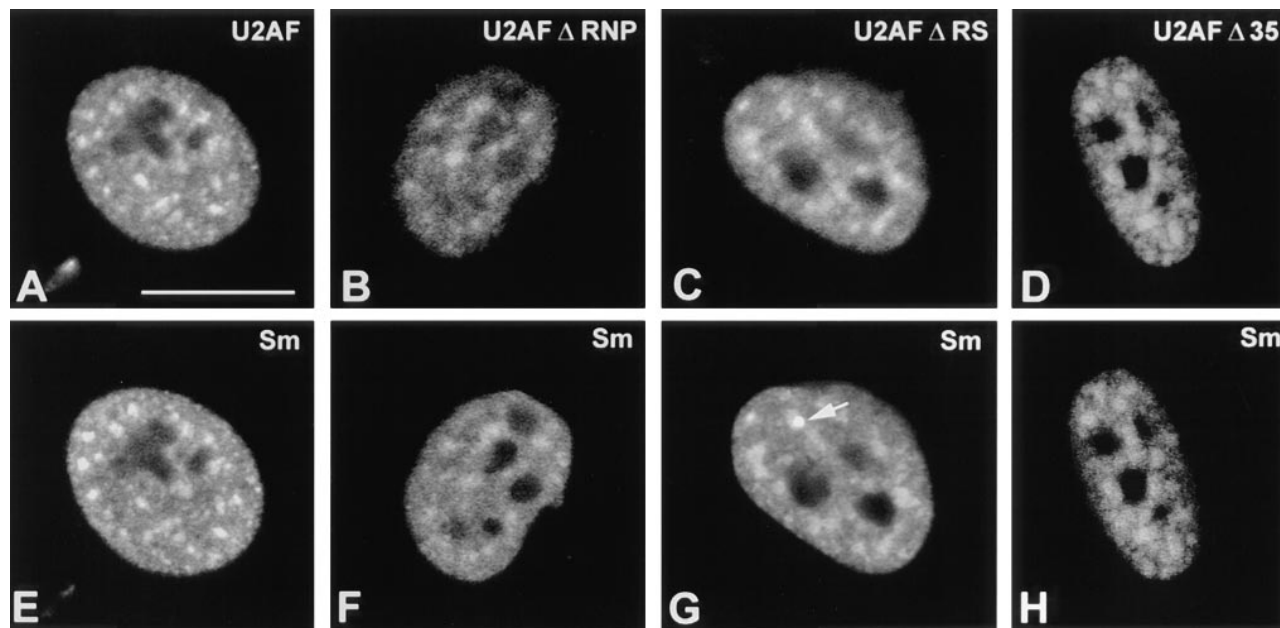
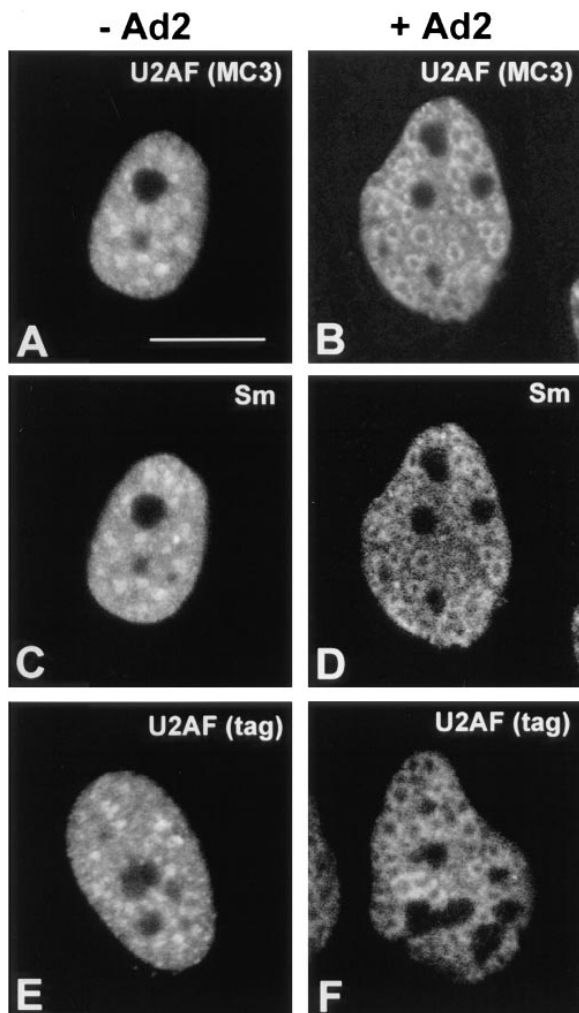


Figure 8. Epitope-tagged recombinant U2AF is targeted to nuclear speckles. HeLa cells were transfected with either U2AF⁶⁵-T (A and E), U2AF⁶⁵ΔRNP2,3-T (B and F), U2AF⁶⁵ΔRS-T (C and G), or U2AF⁶⁵Δ84-150-T (D and H). Cells were fixed at 24 to 36 h after transfection, and the ectopically expressed protein was detected using mAb 12CA5 directed against the HA tag. Double labeling experiments were performed using mAb 12CA5 (A–D) and anti-Sm antibody (E–H). An arrow in G indicates a coiled body that is stained by anti-Sm antibody but not by mAb 12CA5. Cells depicted in A–C and E–G were permeabilized with Triton X-100 before fixation, whereas the cells depicted in D and H were first fixed with formaldehyde and then permeabilized with Triton X-100. Extraction with detergent before fixation significantly enhanced the speckled staining pattern. To ensure that U2AF was also detected in speckles when cells were first fixed and then extracted with detergent, some samples were treated with actinomycin D for 1 h before fixation (D and H). As previously demonstrated for other splicing factors, actinomycin D induces an accumulation of U2AF⁶⁵ in enlarged, rounded up speckles. Bar, 10 μm.



inantly observed in enlarged speckles that also contain splicing snRNPs, SR protein splicing factors, and viral RNA (data not shown; see Bridge et al., 1996).

Consistent with the idea that upon viral infection, U2AF⁶⁵ is recruited from the nuclear speckles to ring-like structures that represent sites of active viral RNA transcription and processing, treatment of cells infected with adenovirus for 14–16 h with transcription inhibitors such as actinomycin D or DRB induces a relocation of U2AF⁶⁵ into speckles (Fig. 10, A and D, B and E). After removal of DRB, U2AF⁶⁵ is again detected in ring-like structures associated with the viral genomes (Fig. 10, C and F), similar to the pattern observed in untreated cells (Figs. 10 C and 9, B and F).

To test whether any of the major functional domains of U2AF⁶⁵ is required for targeting the protein to viral splicing sites, HeLa cells were transfected with either U2AF⁶⁵ΔRS-T, U2AF⁶⁵ΔRNP2,3-T, U2AF⁶⁵Δ84-150-T, or U2AF⁶⁵Δ84-260-T and then infected with adenovirus (Fig. 11). The ectopically expressed proteins were detected us-

Figure 9. Adenovirus induces a redistribution of U2AF⁶⁵ in the nucleus of infected cells. HeLa cells were either mock infected (A, C, and E) or infected with Ad2 for 18 h, at a multiplicity of infection of 20 focus forming units per cell (B, D, and F). The cells were permeabilized with Triton X-100, fixed with formaldehyde, and double labeled using mAb MC3 and anti-Sm antibody (A and C, B and D). Alternatively, cells were first transfected with U2AF⁶⁵WT-T and then were either mock infected or infected with Ad2 (E and F). The ectopically expressed protein was detected using mAb 12CA5. Note that in uninfected cells, all antibodies produce a speckled staining of the nucleoplasm (A, C, and E), whereas after infection the staining is concentrated in ring-like structures (B, D, and F). Bar, 10 μm.

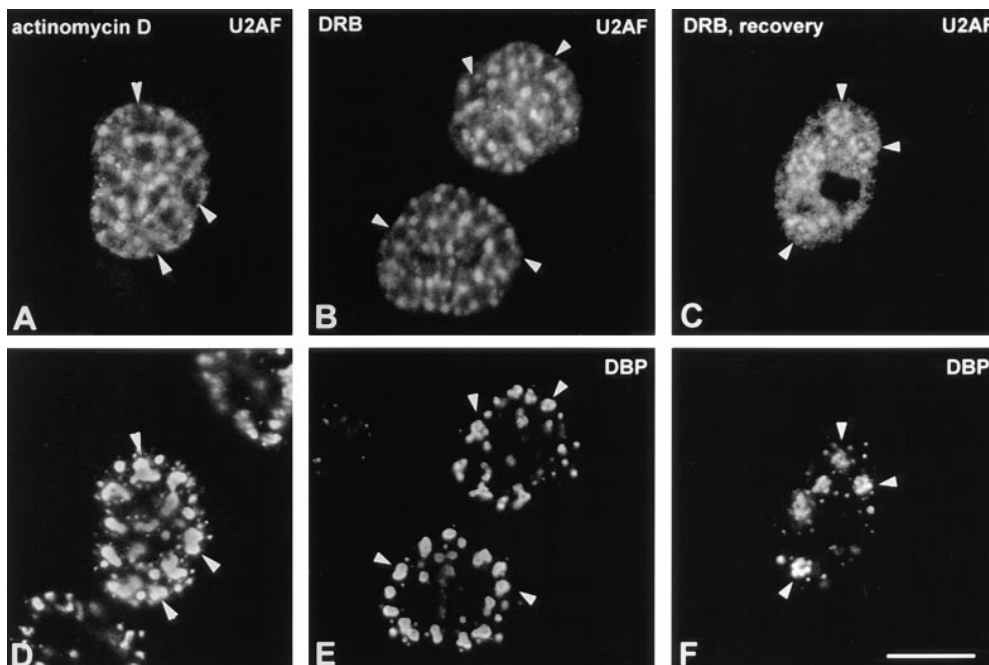


Figure 10. U2AF⁶⁵ relocates to speckles upon inhibition of adenoviral transcription. HeLa cells were transiently transfected with HA-tagged U2AF⁶⁵ for 24 h and then infected with Ad2. At 14 h after infection the cells were treated with either 5 μg/ml actinomycin D (A and D) or 75 μM DRB for 1 h (B and E). After DRB treatment, the drug was removed from the culture medium, and the cells were allowed to recover for 1 h (C and F). A–C depict U2AF⁶⁵ labeling using an anti-tag antibody (mAb 12C5), and panels D–F depict the sites of viral genome accumulation using an antibody directed against the viral DNA binding protein DBP. Arrowheads point to the localization of viral centers. Bar, 10 μm.

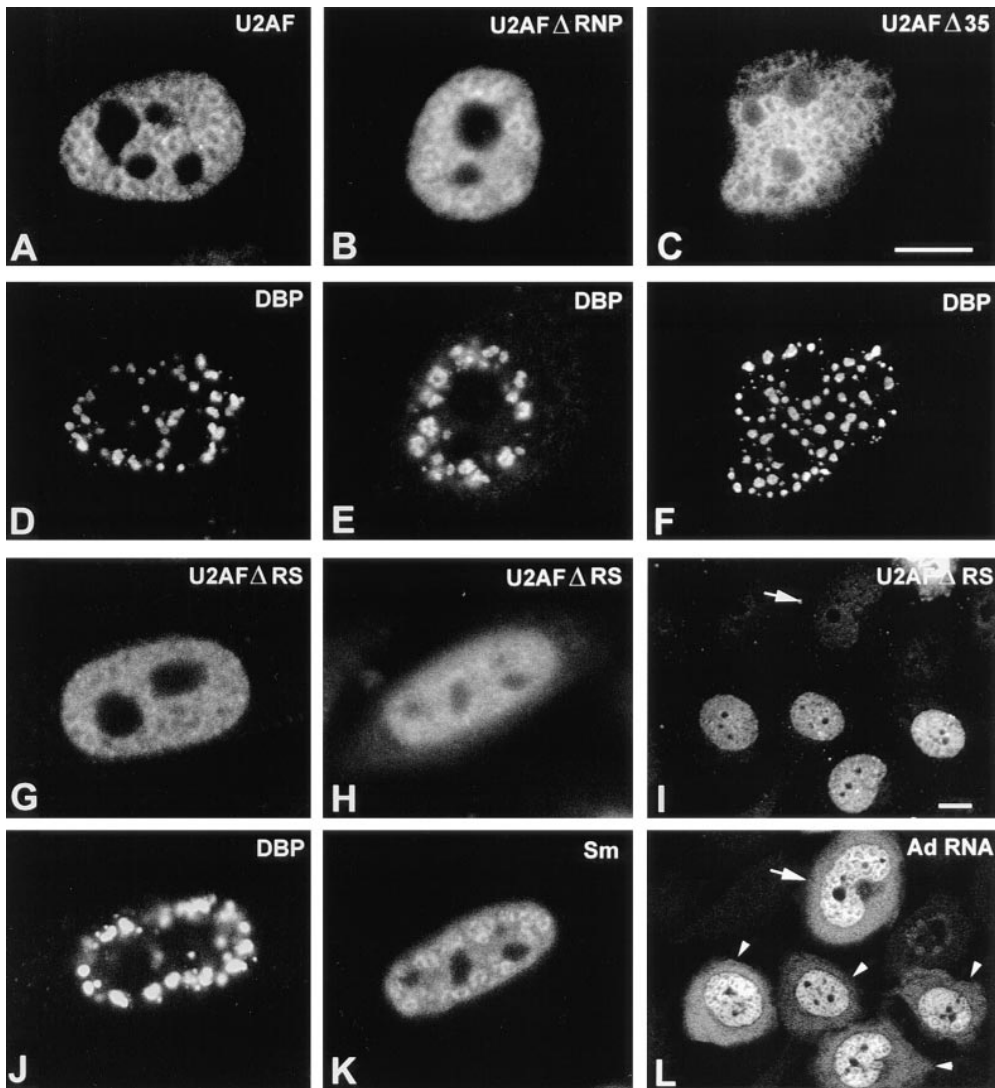


Figure 11. Recruitment of U2AF⁶⁵ to adenoviral splicing sites. HeLa cells were transiently transfected with either U2AF⁶⁵WT-T (A and D), U2AF⁶⁵ΔRNP2,3-T (B and E), U2AF⁶⁵Δ84-150-T (C and F), or U2AF⁶⁵ΔRS-T (G-L) for 24-48 h and then infected with Ad2 and harvested at 18 h after infection. The cells were permeabilized with Triton X-100, fixed with formaldehyde, and double labeled using mAb 12C5 (A-C and G) and a rabbit polyclonal antibody directed against the viral DNA binding protein DBP (D-F and J). Alternatively, the cells were double labeled with mAb 12C5 and anti-Sm antibody (H and K). The cells depicted in I and L were labeled with mAb 12C5, digested with DNaseI, and hybridized with an Ad2 genomic probe to detect viral RNA as previously described (Pombo et al., 1994). Note that cells expressing U2AF⁶⁵ΔRS contain viral RNA in both the nucleus and the cytoplasm (arrowheads); the arrow indicates a nontransfected cell. Similar results were observed in cells hybridized with a splice junction oligonucleotide probe (Bridge et al., 1996) to detect spliced Ad2 RNA (not shown). Bars, 10 μm.

ing the anti-tag mAb 12CA5, and the viral genomes were visualized using a polyclonal antibody specific for the DBP as previously described (Pombo et al., 1994). The results confirm that U2AF⁶⁵WT-T concentrates around the sites of viral genome accumulation (Fig. 11, A and D). Unexpectedly, the deletion mutant U2AF⁶⁵ΔRNP2,3-T, which does not bind to pre-mRNA *in vitro* (Zamore et al., 1992), is also redistributed to the sites of viral genome accumulation and transcription (Fig. 11, B and E), indicating that binding of U2AF⁶⁵ to pre-mRNA is not essential to recruit the protein to splicing sites *in vivo*, and similar results were observed with U2AF⁶⁵Δ84-150-T (Fig. 11, C and F) and U2AF⁶⁵Δ84-260-T (data not shown). In contrast, the deletion mutant U2AF⁶⁵ΔRS-T fails to concentrate in the typical ring structures (Fig. 11, G and J). As the U2AF⁶⁵ RS region is essential for splicing (Zamore et al., 1992; Valcárcel et al., 1996), expression of the U2AF⁶⁵ΔRS-T construct could exert a dominant negative effect and therefore inhibit splicing in these cells. Consequently, the absence of ring structures could reflect a block in splicing activity. To address this point, cells were transfected with U2AF⁶⁵ΔRS-T,

infected with adenovirus, and double labeled with anti-tag and anti-Sm antibodies (Fig. 11, H and K). As depicted in Fig. 11 K, Sm proteins are localized in ring structures, indicating that expression of the U2AF⁶⁵ΔRS-T construct does not prevent recruitment of splicing snRNPs to the sites of viral RNA synthesis. Furthermore, cells that express the U2AF⁶⁵ΔRS-T construct are shown to contain spliced viral RNA in both the nucleus and the cytoplasm (Fig. 11, I and L, and data not shown). This implies that normal processing/transport of adenoviral RNA is taking place in these cells.

In conclusion, the data suggest that the RS domain of U2AF⁶⁵ may play an important role in targeting the protein to sites of active transcription and splicing *in vivo*.

Discussion

In this study we show that a novel monoclonal antibody specific for the splicing factor U2AF⁶⁵ produces a diffuse staining throughout the nucleoplasm with additional concentration in nuclear speckles, and a similar distribution

pattern is observed in cells transiently transfected with epitope-tagged recombinant U2AF⁶⁵. This makes it likely that U2AF⁶⁵ is localized to nuclear speckles.

Within the nucleus, U2AF⁶⁵ is shown to colocalize with splicing snRNPs and SR protein splicing factors in speckles that correspond to clusters of interchromatin granules at the electron microscopic level. Since these clusters also contain poly (A) RNA (Carter et al., 1991; Visa et al., 1993), the presence of splicing factors in these structures has been thought to represent a subnuclear compartmentalization of spliceosomes assembled on pre-mRNA (Carter et al., 1993). However, the findings that splicing occurs co-transcriptionally (Beyer and Osheim, 1988; LeMaire and Thummel, 1990; Bauren and Wieslander, 1994) and that there is no transcriptional activity in clusters of interchromatin granules (Fakan, 1994) argue against the idea that speckles represent splicing sites. Alternatively, clusters of interchromatin granules may be implicated in storage or pre-assembly of the splicing machinery (Spector, 1993; Zhang et al., 1994). Here we present further evidence consistent with this view. First, U2AF⁶⁵ is concentrated in interchromatin granules during mitosis when transcription and splicing are downregulated. Second, at the end of mitosis, U2AF⁶⁵ is targeted to clusters of interchromatin granules in the absence of newly synthesised poly (A) RNA in the nucleus. Finally, the deletion mutant U2AF⁶⁵ΔRS, which fails to be recruited to active splicing sites, maintains the ability to localize in nuclear speckles.

Although U2AF⁶⁵ colocalizes with SR protein splicing factors in nuclear speckles, there are some differences in the subnuclear distribution of these two types of proteins. First, during interphase, U2AF⁶⁵ is clearly widespread in the nucleoplasm with additional concentration in speckles, whereas SR proteins are predominantly detected in speckles. Second, at the end of mitosis, U2AF⁶⁵ is rapidly imported into the daughter cell nuclei, while SR proteins remain concentrated in large cytoplasmic speckles for a longer period of time. Thus, the intracellular dynamics of U2AF⁶⁵ appear to be more similar to snRNPs than to SR proteins, and this discrepancy may be related to the structural and functional differences between U2AF⁶⁵ and SR proteins. In fact, U2AF⁶⁵ binds to the polypyrimidine tract of pre-mRNA and promotes binding of U2 snRNP to the branchpoint (Ruskin et al., 1988; Zamore and Green, 1989), whereas SR protein splicing factors are implicated in 5' and 3' splice site selection (Fu and Maniatis, 1992; Zahler et al., 1992; Wu and Maniatis, 1993). Particularly, the RS region of U2AF⁶⁵ promotes a base-pairing interaction between U2 snRNA and the pre-mRNA (Valcárcel et al., 1996), while the RS domains of SR protein splicing factors mediate protein-protein interactions in the spliceosome (Wu and Maniatis, 1993; Amrein et al., 1994; Kohtz et al., 1994).

In addition to U2AF⁶⁵, U2AF³⁵, and SR protein splicing factors, RS domains are also present in other proteins involved in splicing. These include the U1 snRNP 70-kD protein and the *Drosophila* splicing regulators suppressor of white-apricot, Transformer (Tra), and Transformer 2 (Tra 2). Significantly, all of these proteins localize to nuclear speckles in mammalian cells (Verheijen et al., 1986; Li and Bingham, 1991; Hedley et al., 1995), and recent evidence indicates that an amino acid sequence within the RS

domain of the *Drosophila* Tra protein is sufficient for targeting an heterologous protein to nuclear speckles (Li and Bingham, 1991; Hedley et al., 1995). Like Tra, U2AF⁶⁵ contains an RS domain with a stretch of basic amino acids followed by RS dipeptides and a putative bipartite NLS (Fig. 6 A), and we show here that it is also present in nuclear speckles. However, our results further indicate that the intranuclear distribution of the protein is not affected by deletion of the RS domain. In this regard, it is important to note that a Tra deletion mutant lacking the "speckle localization signal" has also been shown to localize to speckles, presumably through interactions with another protein (Tra 2) that contains the signal (Hedley et al., 1995). Thus, it is conceivable that protein-protein interactions may be responsible for targeting the U2AF⁶⁵ΔRS mutant to the speckles, and a potential candidate is U2AF³⁵. In fact, the RS domain of U2AF³⁵ may also contain a putative "speckle localization signal" (Fig. 6 A), and it binds to a region of U2AF⁶⁵ that is conserved in U2AF⁶⁵ΔRS. Interestingly, deletion of the U2AF⁶⁵ region that interacts with U2AF³⁵ also does not affect targeting to the speckles, indicating that this part of the protein is not essential for the subnuclear localization of U2AF⁶⁵. However, it remains possible that either the presence of an RS domain in the protein or interaction with U2AF³⁵ may be sufficient to target U2AF⁶⁵ to the speckles, and further mutagenesis work is currently in progress to address this question. Also, more immunolocalization studies are necessary to clarify the subnuclear distribution of U2AF³⁵ since previous reports have failed to detect this protein in speckles (Zhang et al., 1992).

In addition to the repeating RS dipeptides and a stretch of basic amino acids, the RS region of U2AF⁶⁵ contains a potential nuclear localization signal (Fig. 6), and therefore one might expect that the deletion mutant U2AF⁶⁵ΔRS would not be transported into the nucleus. Our observation that this mutant is present in the nucleus and localizes to speckles raises the possibility that protein-protein interactions may play an important role in both nuclear and subnuclear localization of U2AF, as recently pointed out by Hedley et al. (1995) for other splicing proteins.

Previous studies have demonstrated that adenovirus subverts the normal organization of splicing factors in the mammalian cell nucleus, although different patterns of distribution have been described depending on the infection stage. When infected cells are treated with a DNA synthesis inhibitor to block viral replication (Zhang et al., 1994) or in cells analyzed at an early stage of infection (Gama-Carvalho, M., R.D. Krauss, L. Chiang, J. Valcárcel, M.R. Green, and M. Carmo-Fonseca, unpublished results), splicing snRNPs are localized in nuclear speckles that do not associate with sites of viral transcription and splicing. After the onset of viral replication, at 14–18 h after infection, snRNPs redistribute from the speckles to ring-like structures located at the periphery of the viral genomes and which have been shown to correspond to sites of viral RNA synthesis (Pombo et al., 1994). At later stages of infection (20–24 h after infection), snRNPs and splicing factors are predominantly detected in large speckles that contain spliced adenoviral RNA and represent enlarged clusters of interchromatin granules (Bridge et al., 1993, 1995, 1996; Puvion-Dutilleul et al., 1994). Here we have

analyzed cells at intermediate stages of adenoviral infection when sites of active viral transcription and splicing can be easily identified as ring structures, and we show that U2AF⁶⁵ colocalizes with splicing snRNPs in these structures. Furthermore, upon treatment of infected cells with transcription inhibitors, U2AF⁶⁵ was predominantly detected in speckles and not in ring structures, whereas after release of the drug, U2AF⁶⁵ was again detected in rings and not in speckles. This suggests that at intermediate stages of infection adenovirus induces a recruitment of U2AF⁶⁵ from the nuclear speckles to sites of active splicing, and we have made use of this model system to test whether deletion mutations that render U2AF⁶⁵ unable to support splicing in vitro also affect recruitment to spliceosomes in vivo. The results show that the U2AF⁶⁵ΔRNP2,3 deletion mutant, which does not bind to pre-mRNA in vitro (Zamore et al., 1992), the U2AF⁶⁵Δ84-150 mutant, which has a deletion covering the region of interaction with U2AF³⁵, and U2AF⁶⁵Δ84-260, which has a deletion spanning both the region of interaction with U2AF³⁵ and the RNP1 domain, are all recruited to the sites of viral splicing. In contrast, the U2AF⁶⁵ΔRS mutant fails to concentrate in the ring structures that represent the sites of adenoviral RNA synthesis. This raises the possibility that in vivo the RS region of U2AF⁶⁵ may be implicated in interactions that are sufficient to recruit the protein to sites of active transcription and splicing, even in the absence of RNA binding capacity.

We wish to acknowledge Professor David-Ferreira for support. We are also grateful to Mr. João Romão for animal care facilities, to Professors M. Lafarga and M.T. Berciano for testing mAb MC3 on cryosections from brain tissue, and to Dr. Fátima Almeida for help with microinjection experiments. We thank the following groups for generously providing materials used in this study: Professor A. Lamond, Dr. J. Mermoud, and Dr. K. Bohmann for SR protein extract, anti-coilin antibody 204, and poly (U) riboprobe; Dr. Eileen Bridge for adenoviruses, splice-junction probes, and anti-DBP antibodies; Professor Walther van Venrooij for anti-Sm autoimmune serum C45; Professor G. Dreyfuss for anti-hnRNP A1 antibody 4B10; and Prof. B. Turner for 3C5 antibody.

This study was supported by grants from Junta Nacional de Investigação Científica e Tecnológica /Program PRAXIS XXI. M. Gama-Carvalho was supported by a PRAXIS XXI research fellowship, and R. Krauss was the recipient of a post-doctoral fellowship from the American Cancer Society.

References

Amrein, H., M.L. Hedley, and T. Maniatis. 1994. The role of specific protein-RNA and protein-protein interactions in positive and negative control of pre-mRNA splicing by the Transformer 2. *Cell*. 76:735-746.

Andersson, S., D.C. Davis, H. Dahlback, H. Jornvall, and P. W. Russel. 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 26 hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* 264:8222-8229.

Baserga, S.J., and J.A. Steitz. 1993. The diverse world of small ribonucleoproteins. In *The RNA World*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 359-385.

Bauren, G., and L. Wieslander. 1994. Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. *Cell*. 76:183-192.

Beyer, A.L., and Y.N. Osheim. 1988. Splice site selection, rate of splicing and alternative splicing on nascent transcripts. *Genes Dev.* 2:754-765.

Bohmann, K., J. Ferreira, N. Santama, K. Weis, and A.I. Lamond. 1995a. Molecular analysis of the coiled body. *J. Cell Sci.* 19(Suppl.):107-113.

Bohmann, K., J.A. Ferreira, and A.I. Lamond. 1995b. Mutational analysis of p80 coilin indicates a functional interaction between coiled bodies and the nucleolus. *J. Cell Biol.* 131:817-831.

Bridge, E., M. Carmo-Fonseca, A.I. Lamond, and U. Pettersson. 1993. Nuclear organization of splicing snRNPs in adenovirus infected cells. *J. Virol.* 67:5792-5802.

Bridge, E., D.X. Xia, M. Carmo-Fonseca, B. Cardinali, A.I. Lamond, and U.

Pettersson. 1995. Dynamic organization of splicing factors in adenovirus-infected cells. *J. Virol.* 69:281-290.

Bridge, E., K.-U. Riedel, B.-M. Johansson, and U. Pettersson. 1996. Spliced exons of adenovirus late RNAs colocalize with snRNP in a specific nuclear domain. *J. Cell Biol.* 135:303-314.

Burd, C.G., and G. Dreyfuss. 1994. Conserved structures and diversity of functions of RNA-binding proteins. *Science (Wash. DC)*. 265:615-621.

Carmo-Fonseca, M., D. Tollervey, R. Pepperkok, S.M.L. Barabino, A. Merdes, C. Brunner, P.D. Zamore, M.R. Green, E. Hurt, and A.I. Lamond. 1991. Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:195-206.

Carmo-Fonseca, M., R. Pepperkok, M.T. Carvalho, and A.I. Lamond. 1992. Transcription-dependent colocalization of the U1, U2, U4/U6 and U5 snRNPs in coiled bodies. *J. Cell Biol.* 117:1-14.

Carter, K.C., K.L. Taneja, and J.B. Lawrence. 1991. Discrete nuclear domains of poly(A)RNA and their relationship to the functional organization of the nucleus. *J. Cell Biol.* 115:1191-1202.

Carter, K.C., D. Bowman, W. Carrington, K. Fogarty, J.A. McNeil, F.S. Fay, and J.B. Lawrence. 1993. A three-dimensional view of precursor messenger RNA metabolism within the mammalian nucleus. *Science (Wash. DC)*. 259:1330-1334.

Dignam, J.D., R.M. Lebowitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475-1489.

Fakan, S. 1994. Perichromatin fibrils are in situ forms of nascent transcripts. *Trends Cell Biol.* 4:86-90.

Ferreira, J.A., M. Carmo-Fonseca, and A.I. Lamond. 1994. Differential interaction of splicing snRNPs with coiled bodies and interchromatin granules during mitosis and assembly of daughter cell nuclei. *J. Cell Biol.* 126:11-23.

Fey, E.G., G. Krochmalnic, and S. Penman. 1986. The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* 102:1654-1665.

Fu, X.-D., and T. Maniatis. 1990. Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature (Lond.)*. 343:437-441.

Fu, X.-D., and T. Maniatis. 1992. Isolation of a complementary DNA that encodes the mammalian splicing factor SC-35. *Science (Wash. DC)*. 256:535-538.

Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hedley, M.L., H. Amrein, and T. Maniatis. 1995. An amino-acid sequence motif sufficient for subnuclear localization of an arginine/serine-rich splicing factor. *Proc. Natl. Acad. Sci. USA*. 92:11524-11528.

Jiménez-García, L.F., and D.L. Spector. 1993. In vivo evidence that transcription and splicing are coordinated by a recruiting mechanism. *Cell*. 73:47-59.

Kiledjian, M., C.G. Burd, D.S. Portman, and G. Dreyfuss. 1994. Structure and function of hnRNP proteins. In *RNA-protein interactions: Frontiers in Molecular Biology*. K. Nagai and I.W. Mattaj, editors. IRL Press, Oxford, UK. 127-149.

Kohtz, J.D., S.F. Jamison, C.L. Will, Z. Ping, R. Luhrmann, M.A. Garcia-Blanco, and J.L. Manley. 1994. Protein-protein interactions and 5'-splice-site recognition in mammalian mRNA precursors. *Nature (Lond.)*. 368:119-124.

Krämer, A. 1996. The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu. Rev. Biochem.* 65:367-409.

Lamm, G.M., and A.I. Lamond. 1993. Non-snRNP protein splicing factors. *Biochim. Biophys. Acta*. 1173:247-265.

LeMaire, M.F., and C.S. Thummel. 1990. Splicing precedes polyadenylation during *Drosophila* E74A transcription. *Mol. Cell. Biol.* 10:6059-6063.

Li, H., and P.M. Bingham. 1991. Arginine/serine-rich domains of the Su(w^e) and tra RNA processing regulators target proteins to a subnuclear compartment implicated in splicing. *Cell*. 67:335-342.

Linné, T.H., H. Jornvall, and L. Philipson. 1977. Purification and characterization of the phosphorylated DNA binding protein from adenovirus type 2 infected cells. *Eur. J. Biochem.* 76:481-490.

Mayeda, A., A.M. Zahler, A.R. Krainer, and M.B. Roth. 1992. Two members of a conserved family of nuclear phosphoproteins are involved in pre-mRNA splicing. *Proc. Natl. Acad. Sci. USA*. 89:1301-1304.

Monneron, A., and W. Bernhard. 1969. Fine structural organization of the interphase nucleus in some mammalian cells. *J. Ultrastruct. Res.* 27:266-288.

Moore, M.J., C.C. Query, and P. Sharp. 1993. Splicing of precursors to mRNA by the spliceosome. In *The RNA World*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 303-357.

Piñol-Roma, S., and G. Dreyfuss. 1991. Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins. *Science (Wash. DC)*. 253:312-314.

Piñol-Roma, S., Y.D. Choi, M.J. Matunis, and G. Dreyfuss. 1988. Immunopurification of heterogeneous nuclear ribonucleoprotein particles reveals an assortment of RNA-binding proteins. *Genes Dev.* 2:215-227.

Pombo, A., J. Ferreira, E. Bridge, and M. Carmo-Fonseca. 1994. Adenovirus replication and transcription sites are spatially separated in the nucleus of infected cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:5075-5085.

Prescott, D.M., and M.A. Bender. 1962. Synthesis of RNA and protein during mitosis in mammalian tissue culture cells. *Exp. Cell Res.* 26:260-268.

Puvion-Dutilleul, F., J.P. Bachelier, N. Visa, and E. Puvion. 1994. Rearrange-

- ments of intranuclear structures involved in RNA processing in response to adenovirus infection. *J. Cell Sci.* 107:1457–1468.
- Rosbash, M., and R.H. Singer. 1993. RNA travel: tracks from DNA to the cytoplasm. *Cell.* 75:399–401.
- Rudner, D.Z., R. Kanaar, K.S. Breger, and D.C. Rio. 1996. Mutations in the small subunit of the *Drosophila* U2AF splicing factor cause lethality and developmental defects. *Proc. Natl. Acad. Sci. USA.* 93:10333–10337.
- Ruskin, B., P.D. Zamore, and M.R. Green. 1988. A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. *Cell.* 52:207–219.
- Singh, R., J. Valcárcel, and M.R. Green. 1995. Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. *Science (Wash. DC).* 268:1173–1176.
- Spector, D.L. 1993. Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.* 9:265–315.
- Spector, D.L., X.-D. Fu, and T. Maniatis. 1991. Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3467–3481.
- Sproat, B.S., A.I. Lamond, B. Beijer, P. Neuner, and U. Ryder. 1989. Highly efficient chemical synthesis of 2'-O-methyl oligoribonucleotides and tetrabiotinylated derivatives: novel probes that are resistant to degradation by RNA or DNA specific nucleases. *Nucleic Acids Res.* 17:3372–3386.
- Turner, B.M., and L. Franchi. 1987. Identification of protein antigens associated with the nuclear matrix and with clusters of interchromatin granules in both interphase and mitotic cells. *J. Cell Sci.* 87:269–282.
- Valcárcel, J., R. Singh, P.D. Zamore, and M.R. Green. 1993. The protein sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. *Nature (Lond.)*. 362:171–175.
- Valcárcel, J., R.K. Gaur, R. Singh, and M.R. Green. 1996. Interaction of U2AF⁶⁵ RS region with pre-mRNA of branch point and promotion base pairing with U2 snRNA. *Science (Wash. DC).* 273:1706–1709.
- Verheijen, R., H. Kuijpers, P. Vooijs, W. VavVenrooij, and F. Ramaekers. 1986. Distribution of the 70K U1 RNA-associated protein during interphase and mitosis. Correlation with other U RNP particles and proteins of the nuclear matrix. *J. Cell Sci.* 86:173–190.
- Visa, N., F. Puvion-Dutilleul, F. Harper, J.P. Bachellerie, and E. Puvion. 1993. Intranuclear distribution of poly(A) RNA determined by electron microscope in situ hybridization. *Exp. Cell Res.* 208:19–34.
- Wu, J.Y., and T. Maniatis. 1993. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell.* 75:1061–1070.
- Zahler, A.M., W.S. Lane, J.A. Stolk, and M.B. Roth. 1992. SR proteins: a conserved family of pre-mRNA splicing factors. *Genes Dev.* 6:837–847.
- Zamore, P.D., and M.R. Green. 1989. Identification, purification and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor. *Proc. Natl. Acad. Sci. USA.* 86:9243–9247.
- Zamore, P.D., and M.R. Green. 1991. Biochemical characterization of U2 snRNP auxiliary factor: an essential pre-mRNA factor with a novel intranuclear distribution. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:207–214.
- Zamore, P.D., J.G. Patton, and M.R. Green. 1992. Cloning and domain structure of the mammalian splicing factor U2AF. *Nature (Lond.)*. 355:609–614.
- Zhang, G., P.D. Zamore, M. Carmo-Fonseca, A.I. Lamond, and M.R. Green. 1992. Cloning and intracellular localization of the U2 small nuclear ribonucleoprotein auxiliary factor small subunit. *Proc. Natl. Acad. Sci. USA.* 89:8769–8773.
- Zhang, G., K.L. Taneja, R.L. Singer, and M.R. Green. 1994. Localization of pre-mRNA splicing in mammalian nuclei. *Nature (Lond.)*. 372:809–812.
- Zuo, P., and T. Maniatis. 1996. The splicing factor U2AF35 mediates critical protein-protein interactions in constitutive and enhancer-dependent splicing. *Genes Dev.* 10:1356–1368.