Protein 4.1 was originally described as an 80 kDa polypeptide present in cytoskeletal preparations of mature human red blood cells. Protein 4.1 forms a ternary complex with erythrocyte spectrin and short filaments of actin, and thereby amplifies the interaction of the latter two proteins. The formation of the spectrin-actin-4.1 ternary complex is essential for the maintenance of normal red blood cell shape. Thus, duplication or deletion of the spectrin-actin binding site of protein 4.1, located within a 65 amino acid fragment near the COOH-terminal region of the molecule (Correas et al., 1986a,b), is associated with congenital hemolytic anemias (Delaunay, 1995). Via interactions with integral membrane proteins including band 3 and glycophorin A and C, protein 4.1 establishes a link between the spectrin-based cytoskeleton and the plasma membrane (reviewed by Conboy, 1993).

Numerous isoforms of protein 4.1 presenting a variety of sizes that range from 30 kDa to 210 kDa have been identified in erythroid as well as in nonerythroid tissues (Anderson et al., 1988; Granger and Lazarides, 1984). Posttranslational modifications partly account for the impressive diversity of 4.1 proteins; however, alternative splicing of the 4.1 pre-mRNA and the existence of two possible initiation codons have been suggested as major causes for the generation of multiple 4.1 isoforms (reviewed by Conboy, 1993). Interestingly, the expression of some 4.1 isoforms appears to be tissue-specific and developmentally regulated (Conboy, 1993; Granger and Lazarides, 1984; Tang et al., 1988; Baklouti et al., 1996). Besides the many different 4.1 proteins described in nonerythroid cells, very little is known about their possible functions. The presence of 4.1 in stress fibers (Cohen et al., 1982), in perinuclear regions (Leto et al., 1986) as well as in the nucleus (Correas, 1991; De Cárcer et al., 1995; Lallena and Correas, 1997; Krauss et al., 1997) suggests that nonerythroid 4.1 proteins also play roles different from attaching the cytoskeletal scaffold to the membrane.

In mammalian cells, pre-mRNA splicing proteins mainly accumulate in two nuclear domains referred to as ‘speckles’, which exist at the level of 20-50 per nucleus (reviewed by Spector, 1993), and ‘coiled bodies’, which exist at the level of one to five (reviewed by Lamond and Carmo-Fonseca, 1993). Both structures are enriched in U2, U4/6, and U5 small nuclear ribonucleoprotein particles (snRNPs) and also non-snRNP splicing factors. The localization of components of the splicing machinery in the nucleus has been demonstrated to be highly dynamic and influenced by a number of factors; thus, inhibition of RNA polymerase II provokes a redistribution of the splicing factor SC35. Based on our combined biochemical and localization results, we propose that 4.1 proteins are part of nuclear structures to which splicing factors functionally associate, most likely for storage purposes.

Key words: Protein 4.1, Splicing, SC35, snRNP, SR protein, U2AF35, U2AF65

**SUMMARY**

Protein 4.1 is a multifunctional polypeptide that links transmembrane proteins with the underlying spectrin/actin cytoskeleton. Recent studies have shown that protein 4.1 is also present in the nucleus, localized in domains enriched in splicing factors. Here we further analyze the relationship between protein 4.1 and components of the splicing machinery. Using HeLa nuclear extracts capable of supporting the splicing of pre-mRNAs in vitro, we show that anti-4.1 antibodies specifically immunoprecipitate pre-mRNA and splicing intermediates. Immunodepletion of protein 4.1 from HeLa nuclear extracts results in inhibition of their splicing activity, as assayed with two different pre-mRNA substrates. Coprecipitation of protein 4.1 from HeLa nuclear extracts with proteins involved in the processing of pre-mRNA further suggests an association between nuclear protein 4.1 and components of the splicing apparatus. The molecular cloning of a 4.1 cDNA encoding the isoform designated 4.1E has allowed us to show that this protein is targeted to the nucleus, that it associates with the splicing factor U2AF35, and that its overexpression induces the redistribution of the splicing factor SC35. Based on our combined biochemical and localization results, we propose that 4.1 proteins are part of nuclear structures to which splicing factors functionally associate, most likely for storage purposes.

**INTRODUCTION**

Protein 4.1 was originally described as an 80 kDa polypeptide present in cytoskeletal preparations of mature human red blood cells. Protein 4.1 forms a ternary complex with erythrocyte spectrin and short filaments of actin, and thereby amplifies the interaction of the latter two proteins. The formation of the spectrin-actin-4.1 ternary complex is essential for the maintenance of normal red blood cell shape. Thus, duplication or deletion of the spectrin-actin binding site of protein 4.1, located within a 65 amino acid fragment near the COOH-terminal region of the molecule (Correas et al., 1986a,b), is associated with congenital hemolytic anemias (Delaunay, 1995). Via interactions with integral membrane proteins including band 3 and glycophorin A and C, protein 4.1 establishes a link between the spectrin-based cytoskeleton and the plasma membrane (reviewed by Conboy, 1993).

Numerous isoforms of protein 4.1 presenting a variety of sizes that range from 30 kDa to 210 kDa have been identified in erythroid as well as in nonerythroid tissues (Anderson et al., 1988; Granger and Lazarides, 1984). Posttranslational modifications partly account for the impressive diversity of 4.1 proteins; however, alternative splicing of the 4.1 pre-mRNA and the existence of two possible initiation codons have been suggested as major causes for the generation of multiple 4.1 isoforms (reviewed by Conboy, 1993). Interestingly, the expression of some 4.1 isoforms appears to be tissue-specific and developmentally regulated (Conboy, 1993; Granger and Lazarides, 1984; Tang et al., 1988; Baklouti et al., 1996). Besides the many different 4.1 proteins described in nonerythroid cells, very little is known about their possible functions. The presence of 4.1 in stress fibers (Cohen et al., 1982), in perinuclear regions (Leto et al., 1986) as well as in the nucleus (Correas, 1991; De Cárcer et al., 1995; Lallena and Correas, 1997; Krauss et al., 1997) suggests that nonerythroid 4.1 proteins also play roles different from attaching the cytoskeletal scaffold to the membrane.

In mammalian cells, pre-mRNA splicing proteins mainly accumulate in two nuclear domains referred to as ‘speckles’, which exist at the level of 20-50 per nucleus (reviewed by Spector, 1993), and ‘coiled bodies’, which exist at the level of one to five (reviewed by Lamond and Carmo-Fonseca, 1993). Both structures are enriched in U2, U4/6, and U5 small nuclear ribonucleoprotein particles (snRNPs) and also non-snRNP splicing factors. The localization of components of the splicing machinery in the nucleus has been demonstrated to be highly dynamic and influenced by a number of factors; thus, inhibition of RNA polymerase II provokes a redistribution of the splicing factor SC35. Based on our combined biochemical and localization results, we propose that 4.1 proteins are part of nuclear structures to which splicing factors functionally associate, most likely for storage purposes.

Key words: Protein 4.1, Splicing, SC35, snRNP, SR protein, U2AF35, U2AF65
role in maintaining spatial order within the nucleus. Factors involved in transcription, splicing and replication have been found associated with this structure and although the precise role of the nuclear matrix in organizing these processes is a controversial issue, it is clear that components of the splicing machinery are retained in nuclear matrix preparations (reviewed by van Driel et al., 1995). This indicates that some elements of the nucleoskeleton may be anchoring splicing proteins.

Recently, we have reported that protein 4.1 is a component of the nuclear matrix of mammalian cells and that 4.1 is distributed in speckled domains which have been shown to be enriched in proteins involved in the splicing process (De Cárcer et al., 1995; Lallena and Correas, 1997). In the present study, we analyze further the relationship between protein 4.1 and components of the splicing machinery. We show that: (1) anti-4.1 antibodies immunoprecipitate splicing intermediates and products, (2) nuclear extracts deficient in 4.1 are depleted of their splicing activity, (3) proteins 4.1 present in HeLa nuclear extracts co-immunoprecipitate with proteins involved in the processing of pre-mRNA molecules, (4) a cloned 4.1 cDNA, that encodes an isoform named to as 4.1E, is targeted to the nucleus, (5) protein 4.1E binds to the splicing factor U2AF35, and (6) overexpression of 4.1E induces the redistribution of endogenous SC35. All these results indicate that nuclear protein 4.1 functionally associates with proteins of the splicing apparatus.

MATERIALS AND METHODS

Cell culture, transfection assays and cell treatments

Madin Darby canine kidney (MDCK), HeLa and COS-7 cells were grown on Petri dishes or glass coverslips in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal bovine serum at 37°C, under a 5% CO2 /95% air humidified atmosphere. Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal bovine serum at 37°C, under a 5% CO2 /95% air humidified atmosphere.

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For drug treatments, cells on coverslips were permeabilized with Triton X-100 in PBS for 2 minutes at room temperature and then fixed in 10% formalin (37% formaldehyde solution, Sigma-F61635) for 15 minutes at room temperature. Double-labeling experiments were carried out as described by De Cárcer et al. (1995). Preparations were mounted on microscope slides with Mowiol 40.88 and examined on a Zeiss epifluorescence microscope or on a MRC 1024 confocal laser scanning microscope (Bio-Rad). Controls to assess the specificity and the lack of cross-labeling included incubations with non-immune rabbit serum and irrelevant monoclonal antibodies or omission of either of the primary antibodies.

Splicing assays

Splicing assays were performed in a final volume of 9 μl, with 40% HeLa nuclear extract (Dignam et al., 1983) or depleted extracts, 3.3% polyvinyl-alcohol, 10 fmols of pre-mRNA (20,000 cpm), 3 mM MgCl2, 1 mM ATP, 22 mM creatine-phosphate, 67 mM KCl and 1 U/μl of RNasin. After incubation at 30°C for 60 minutes, RNA was purified by proteinase K digestion, extraction with phenol/chloroform, and precipitation with ethanol. The RNAs were analyzed by electrophoresis on a denaturing 13% polyacrylamide gel.

Immunoprecipitation assays

Affinity-purified anti-4.1 or control anti-tau antibodies (1 μg each) were added to 9 μl of splicing mixture which had been placed on ice after incubation at 30°C for 45 minutes. The reactions were maintained on ice for 15 minutes, and then 20 μl of Protein-A Sepharose beads were added and incubation continued for another 15 minutes. The Protein A-antibody complexes were washed 3 times with 200 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1.5 mM MgCl2 and 0.5 mM DTT. RNA was purified from the immunoprecipitates by treatment with proteinase K, phenol/chloroform extraction and ethanol precipitation, and analyzed by gel electrophoresis on a 13% polyacrylamide denaturing gel.

Immunodepletion of nuclear extracts

Affinity-purified anti-4.1 or control anti-tau antibodies (140 μg each) were immobilized in 100 μl of Protein A-Sepharose beads by incubation for 3 hours at 4°C on a rocking platform. Beads were washed 3 times with 1 ml of nuclear extract buffer (20 mM HEPES, pH 8.0, 20% glycerol, 0.5 mM EDTA, 1 mM DTT and 0.1 M KCN) and incubated with 100 μl of HeLa nuclear extracts for 4 hours at 4°C on a rocking platform. The beads were then separated from the mammalian expression vector (Takebe et al., 1988) and sequenced in an ABI 373A Automatic Sequencer.

Antibodies

The following antibodies were used: affinity-purified polyclonal antibodies directed against the 8-10 kDa domain of protein 4.1 involved in the association of 4.1 with spectrin and actin (Correas et al., 1986a,b); anti-SC35 and anti-c-myc monoclonal antibodies (obtained from the American Type Culture Collection); anti-snRNP monoclonal antibody Y12 (a generous gift from Dr Steitz, Howard Hughes Medical Institute, New Haven, USA). Hybridoma supernatant anti-U2AF65 (a generous gift from Drs Carvalho and Carmino-Fonseca, University of Lisbon, Portugal); anti-SR monoclonal antibody mAb104 (a generous gift from Dr Roth, Fred Hutchinson Cancer Research Center, Seattle, USA); polyclonal anti-nuclear lamin A and C antibodies (generous gifts from Dr Georgatos, University of Crete, Greece). Anti-Tau polyclonal antibody, raised against a synthetic peptide whose sequence corresponds to the tubulin-binding domain on tau molecule (Correas et al., 1990), was used as a control antibody in Figs 3, 4 and 5 at the same concentrations and under the same conditions as the anti-4.1 antibody.

Immunofluorescence and confocal microscopy

Cells grown on coverslips were permeabilized with Triton X-100 in PBS for 2 minutes at room temperature and then fixed in 10% formalin (37% formaldehyde solution, Sigma-F61635) for 15 minutes at room temperature. Double-labeling experiments were carried out as described by De Cárcer et al. (1995). Preparations were mounted on microscope slides with Mowiol 40.88 and examined on a Zeiss epifluorescence microscope or on a MRC 1024 confocal laser scanning microscope (Bio-Rad). Controls to assess the specificity and the lack of cross-labeling included incubations with non-immune rabbit serum and irrelevant monoclonal antibodies or omission of either of the primary antibodies.

RNA extraction and cDNA cloning

Cytoplasmic RNA was extracted from human Molt-4 T cells, as previously described by Favaloro et al. (1980). 4.1 mRNA was specifically reverse-transcribed using AMV reverse transcriptase (Promega) and the specific 4.1 antisense primer corresponding to 3’ untranslated sequences contained in exon 22: CTGAAGTCTGAGAATCCAAGTGGG. The 4.1 cDNAs were then PCR amplified under the conditions described by Conboy et al. (1991) using Taq polymerase (Promega), the specific 4.1 sense primer designated ATG2: CAGCCCGAGCAACATGTA, corresponding to nucleotides 94-112 in the sequence reported by Conboy et al. (1991), and the antisense 4.1-specific primer designated stopE21a: TCACTCATCAGGATCTTGG, corresponding to nucleotides 2,722-2,741. Reaction products were cloned into the pMosBLUE T-vector (Amersham; Buckinghamshire, UK). For expression experiments, 4.1 was tagged at its carboxy terminus with the c-myc 9E10 epitope (MEQKLISEEDL) (Evan et al., 1985) by PCR using the ATG2’ primer (sense) and a primer (antisense) comprising the sequence of the stopE21a primer and additional sequences encoding the c-myc epitope. The PCR product was cloned into the pSRα g gel.
depleted extract by centrifugation at 15,000 rpm for 15 seconds in a microcentrifuge.

**Immunoprecipitation and immunoblotting**

HeLa nuclear extracts (Dignam et al., 1983) were brought to 20 mM Hepes, pH 7.9, 2.5 mM MgCl₂, 100 mM NaCl, 0.1 mM EDTA, 0.05% NP40 and 1.5% Triton X-100 (IP buffer). Protein A-Sepharose beads were incubated with affinity purified anti-4.1 antibodies for 14 hours at 4°C and unbound antibodies were removed by centrifugation. The extracts were incubated with antibody-coupled Sepharose beads for 3 hours at 4°C. The immunoprecipitates were washed four times with IP buffer containing 125 mM, 150 mM, 175 mM and 200 mM NaCl.

Finally, a wash with 40 μl of SDS Laemmli buffer (Laemmli, 1970) containing 0.1 M DTT for 10 minutes at 80°C, microcentrifuged for one minute at room temperature and separated by SDS-PAGE. SR proteins were purified from HeLa cells following the method described by Zahler et al. (1992). Proteins were transferred and developed as described by De Cárcer et al. (1995).

**In vitro-coupled transcription and translation reactions**

Coupled in vitro transcription and translation reactions were done using the TNT T7 reticulocyte lysate system (Promega), and pMos-BLUE cloned 4.1 cDNAs as templates. Synthesized proteins were radiolabeled by including [35 S]methionine (Amersham) in the reaction. Proteins were fractionated by SDS-PAGE and visualized by exposing dried gels to Kodak autoradiographic films.

**RESULTS**

The distribution of nuclear protein 4.1 is highly dynamic

Previous studies from our laboratory have shown that the distribution of protein 4.1 in response to transcriptional inhibitors follows the patterns observed for the splicing factor SC35 (Lallena and Correas, 1997). Here, we extend our previous study by comparing protein 4.1 distribution with that of snRNP antigens.

To induce redistribution of splicing factors, cells were treated either with DRB, an adenosine analogue which inhibits RNA polymerase II transcription partly due to its ability to inhibit protein kinases which phosphorylate the COOH-terminal domain of the largest subunit of the enzyme (Chodosh et al., 1989), or with α-amanitin which inhibits transcription by directly forming a tight complex with RNA polymerase II (Lindell et al., 1970). Cells treated with DRB and stained either with anti-4.1 and anti-snRNP (Fig. 1A,B) or with anti-4.1 and anti-SC35 antibodies (Fig. 1E,F) showed that protein 4.1, as the splicing proteins analyzed, distributed in small and unconnected round speckles. As expected, incubation of cells with α-amanitin resulted in a reorganization of snRNP antigens (Fig. 1D) and SC35 (Fig. 1H) which accumulated in round and enlarged dots lacking apparent interconnections. Protein 4.1 also accumulated in dots (Fig. 1C,G) which appeared superimposable on the splicing-enriched structures (Fig. 1C,D or Fig. 1G,H).

Fig. 2 shows a second set of experiments analyzing the dynamic distribution of nuclear protein 4.1. One advantage of using the transcriptional inhibitor DRB is that it can be rapidly washed out to reverse the transcriptional block (Tamm et al., 1976), thus allowing us to analyze redistribution of splicing proteins. Experiments in which DRB was washed out and cells were maintained at 4°C showed that protein 4.1 (Fig. 2A,E), SC35 (Fig. 2B) and snRNPs (Fig. 2F) distributed in typical round speckles lacking interconnections between them. However, if DRB was washed out and cells were kept at 37°C, protein 4.1 (Fig. 2C,G), SC35 (Fig. 2D) and snRNPs (Fig. 2H) reorganized to irregularly shaped speckles with connections between them, as was observed in control transcriptionally active cells (data not shown).

All these experiments indicate that the distribution of nuclear protein 4.1 is highly dynamic and parallels that of SC35 and snRNPs.

**Immunoprecipitation of splicing intermediates and products by the anti-4.1 antibody**

To further explore the relationship between protein 4.1 and splicing factors, we performed a series of biochemical analysis using HeLa nuclear extracts which are capable of supporting the splicing of pre-mRNAs in vitro (Dignam et al., 1983; Krainer et al., 1984). First, we analyzed whether the anti-4.1 antibody was capable of immunoprecipitating pre-mRNA splicing complexes.

Nuclear extracts were incubated with a 32P-labeled adenovirus pre-mRNA substrate for 45 minutes under conditions that support splicing reactions, after which the anti-4.1 antibody was added and the antibody complexes were immunoprecipitated with Protein-A Sepharose beads. After extensive washing, the RNA present in the

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**Fig. 1.** The distribution of nuclear protein 4.1, as of SC35 and snRNP proteins, is dynamic. MDCK cells were treated with the transcriptional inhibitor DRB (A,B,E,F) or with α-amanitin (C,D,G,H). Analysis of the distribution of 4.1, SC35 and snRNPs was performed by double-immunolabelling with anti-4.1 (A and C) and anti-snRNP (B and D) antibodies or with anti-4.1 (E and G) and anti-SC35 (F and H) antibodies. Protein 4.1 redistributes as SC35 and snRNP proteins do. Bar, 10 μm.
immunoprecipitates was purified and analyzed by electrophoresis in a denaturing gel. Fig. 3 (+ATP) shows that the immunoprecipitates obtained with the anti-4.1 antibody contain pre-mRNA, splicing intermediates, and spliced mRNA (compare lane 1 -input- with lane 2) well above the background of RNA unspecifically attached to the beads (lane 3). Furthermore, when the assay was performed under conditions that do not support pre-mRNA splicing, the levels of immunoprecipitated RNAs obtained with the anti-4.1 antibody were indistinguishable from those immunoprecipitated with the control antibody (Fig. 3, -ATP). As previously observed by others, high amounts of RNA non-specifically associate with the antibody-coated beads in the absence of spliceosome assembly. These results indicate that protein 4.1 is associated with splicing complex components.

Compromised splicing activity of 4.1-immunodepleted nuclear extracts
HeLa nuclear extracts were depleted of 4.1 proteins using affinity-purified antibodies to determine whether or not the removal of 4.1 affects the splicing activity of the extracts.

Undepleted and depleted HeLa nuclear extracts were analyzed by western blotting (Fig. 4A). The anti-4.1 antibody recognized five polypeptides of approximately 25, 55, 80, 105 and 135 kDa in undepleted HeLa nuclear extracts (Fig. 4A, lane 1). Nuclear extracts were quantitatively depleted of the ~25, ~105 and ~135 kDa species and the levels of the 55 and 80 were significantly reduced (Fig. 4A, lane 2). The same depletion procedure with the control antibody produced minor changes in the concentration of these antigens (Fig. 4A, lane 3). Next we tested whether the splicing activity of the nuclear extracts was affected by the depletion of 4.1 proteins. Standard splicing assays were performed with two different model pre-mRNA substrates in either undepleted, mock-depleted or 4.1-depleted nuclear extracts. Fig. 4B and C show that splicing of an adenovirus-major-late (AdML) or a β-globin transcript was compromised in 4.1-depleted nuclear extracts, but not in control-depleted extracts. These experiments indicate that depletion of 4.1 from nuclear extracts can compromise their pre-mRNA splicing activity.

Coprecipitation of components of the splicing machinery with protein 4.1
Since protein 4.1 has been considered as a structural element of the membrane-skeleton of erythrocytes, it is difficult to think
of nuclear 4.1 as a protein directly involved in the process of splicing. It seems more likely that nuclear 4.1 is an element of the nucleoskeleton to which proteins involved in splicing attach. To study this possibility, we performed a series of immunoprecipitation experiments. Anti-4.1 antibodies were first used to immunoprecipitate protein 4.1 (Fig. 5A), and antibodies against proteins involved in splicing were used to check their presence or absence in the immunoprecipitates by western blotting analyses (Fig. 5B to D).

Anti-4.1 antibodies immunoprecipitated three major 4.1 proteins of 80, 105 and 135 kDa from the nuclear extracts used for the assays (Fig. 5A, lane 3), which were not immunoprecipitated by a control antibody (Fig. 5A, lane 4). We tested the presence in the immunoprecipitates of SR proteins, a family of splicing factors that contain a carboxy-terminal domain rich in arginine-serine diaminio acid repeats (Zahler et al., 1992); the presence of Sm epitope-containing components of snRNPs; and the presence of the 3′ splice site-recognizing factor U2AF65 (Zamore and Green, 1991). Our results show that SR proteins (recognized by mAb 104), Sm antigens (recognized by mAb Y12) and U2AF65 (recognized...
by mAb MC3) are present in the material immunoprecipitated by the anti-4.1 antibodies (see Fig. 5B, lane 3; Fig. 5C, lane 2 and Fig. 5D, lane 2, respectively). By contrast, neither lamin A nor C, which are components of the nuclear matrix and are present in the nuclear extracts (Fig. 5E, lane 1), were detected in the immunoprecipitates (Fig. 5E, lane 2). We conclude that protein 4.1 is specifically associated with splicing factors in HeLa nuclear extracts. In agreement with this, immunoprecipitates using mAb104 contained the major 80, 105 and 135 kDa 4.1 proteins (data not shown). In contrast with other antibodies against nuclear matrix proteins (Blencow et al., 1994), the anti-4.1 antibodies do not recognize purified SR proteins (Fig. 5A, lane 2).

Identification of a 4.1 cDNA encoding an isoform that localizes in the nucleus

The facts that: (1) splicing complexes can be immunoprecipitated by the anti-4.1 antibody, (2) the antibody is able to deplete nuclear extracts of their splicing activity, and (3) protein 4.1, SR proteins, snRNPs and the splicing factor U2AF65 coimmunoprecipitate from nuclear extracts, suggest a functional association between nuclear 4.1 and components of the splicing machinery. To get further insight into the association of protein 4.1 with splicing factors, we approached the molecular cloning of 4.1 cDNAs. The nucleated human Molt-4 T-cell line was used as source for isolating 4.1 RNAs; 4.1 cDNAs were obtained by RT-PCR techniques using oligonucleotide primers specific for 4.1 sequences (as described in Materials and Methods). The 4.1 cDNAs were tagged with sequences encoding the c-myc epitope (Evan et al., 1985) to distinguish their protein products from endogenous 4.1 proteins, and cloned into a mammalian expression vector to analyze their intracellular distribution in transfected cells. COS-7 cells transiently expressing epitope-tagged 4.1 proteins were analyzed 48 hours after transfection by immunofluorescence microscopy to analyze their intracellular distribution in transfected cells. COS-7 cells transiently expressing epitope-tagged 4.1 proteins were analyzed 48 hours after transfection by immunofluorescence microscopy using the monoclonal anti-c-myc (9E10) antibody. The results presented in Fig. 6A show that a naturally-occurring protein 4.1, named as 4.1E, has a nuclear localization which resembles that of endogenous nuclear 4.1 proteins as detected in transcriptionally active cells (Correas, 1991; De Cárcer et al., 1995; Lallena and Correas, 1997). The nuclear distribution of 4.1E was consistently observed in >99% out of four hundred transfected cells scored in ten different transfection experiments. No staining was detected with the anti-c-myc antibody in cells transfected with the vector lacking the 4.1E cDNA insert (Fig. 6C).

In vitro and in vivo expression of 4.1E

Protein 4.1E, which is translated from the translation initiation site present in exon 2', comprises exons 2', 2, 6-13, 17, 18-21, and has a predicted size of 78,563 Da. The apparent molecular mass of the in vitro and in vivo synthesized protein 4.1E was determined on SDS-PAGE. 4.1E cDNA was transcribed in vitro with T7 RNA polymerase and translated in vitro using a rabbit reticulocyte lysate. This yielded a product of ~75-80 kDa (Fig. 7A, lane 1), which coincided with its predicted size and with the size of the faster migrating 4.1 band detected in HeLa nuclear extracts. Two products of 65 and 40 kDa were also observed which might correspond to partial 4.1 fragments. None of these products were present in control samples lacking the template 4.1 cDNA (Fig. 7A, lane 2). In agreement with the in vitro results, a protein in the range of ~75-80 kDa was detected with the anti-c-myc antibody in transiently transfected COS cells (lane 2) using anti-c-myc antibody for the immunodetection of the exogenous protein 4.1E. Numbers on the left indicate approximate molecular masses of protein standards in kDa.

Protein 4.1E associates with the splicing factor U2AF35

It is well established that the splicing factor U2AF55 forms a
complex with U2AF\textsuperscript{35} and that their association persists under non-splicing conditions (Zamore and Green, 1989; Zhang et al., 1992). The presence of the splicing factor U2AF\textsuperscript{65} in immunoprecipitates of nuclear extracts led us to investigate the possible interaction between the components of the U2AF complex and 4.1E. To this end, we performed immunoprecipitation assays using a combination of the in vitro translation-products of 4.1E, U2AF\textsuperscript{65} and U2AF\textsuperscript{35} cDNAs. Fig. 8 shows the in vitro synthesized proteins U2AF\textsuperscript{35} (lane 1), translated protein U2AF\textsuperscript{65} (lane 2), translated protein 4.1E (lane 3); a combination of the three translated proteins immunoprecipitated by anti-U2AF\textsuperscript{65} antibody (lane 4); a combination of 4.1E and U2AF\textsuperscript{65} immunoprecipitated by anti-4.1 antibody (lane 5), a combination of 4.1E and U2AF\textsuperscript{35} immunoprecipitated by anti-4.1 antibody (lane 6), immunoprecipitation of U2AF\textsuperscript{35} by anti-4.1 antibody (lane 7). Protein 4.1E associates with the splicing factor U2AF\textsuperscript{35} (lane 6) but not with U2AF\textsuperscript{65} (lane 5).

**Overexpression of protein 4.1E induces SC35 redistribution**

Another line of evidence supporting interactions between 4.1 and splicing factors has been obtained from experiments in which the distribution of overexpressed protein 4.1E has been compared with that of endogenous SC35. Double immunofluorescence and confocal microscopy analyses were performed using polyclonal anti-4.1 and monoclonal anti-SC35 antibodies (Fig. 9). The anti-4.1 antibody was used at dilutions which do not allow detection of endogenous 4.1 proteins (Fig. 9A). Protein SC35 distributes in typical speckles in untransfected cells but, in transfected cells, it concentrates in nuclear domains (Fig. 9B) which are enriched in protein 4.1E (Fig. 9A). An identical redistribution of SC35 from speckles to nuclear loci has been reported by overexpression of U1 snRNP 70K (Romac and Keene, 1995). This result along with the results obtained with inhibitors of RNA pol II (Figs 1 and 2), provides further evidence for the tight colocalization of 4.1 proteins and splicing factors in a variety of cellular situations.

**DISCUSSION**

In erythrocytes, protein 4.1 has been shown to be important for maintenance of the structural integrity and flexibility of the cell membrane and its underlying cytoskeleton. Recent studies have indicated that protein 4.1 is also present in the nucleus, as a component of the nuclear matrix localized in domains enriched in pre-mRNA splicing proteins (De Cárcer et al., 1995; Krauss et al., 1997). Pre-mRNA splicing proteins concentrate in dense nuclear matrix structures which correspond to the speckled domains observed by immunofluorescence. The intranuclear distribution of pre-mRNA splicing proteins is dynamic and changes in their distribution are observed in response to different factors such as heat shock treatment and rate of transcription (Carmo-Fonseca et al., 1992; Spector et al., 1993). Our data indicate that protein 4.1 is subject to similar assembly-disassembly processes as snRNPs and the non-snRNP splicing factor SC35 under situations in which the rate of transcription is altered. Redistribution of nuclear 4.1, as of SC35 and snRNPs, is reversible and temperature dependent suggesting that these proteins respond to a common mechanism.

The nuclear matrix consists of a complex lattice of interconnecting filaments composed of protein and RNA.
This substructure retains snRNPs and various other components of the splicing machinery (reviewed by van Driel et al., 1995). Evidence of an association between splicing and the nuclear matrix was first provided by Zeitlin et al. (1987) who reported that a β-globin pre-mRNA retained on the nuclear matrix was rapidly processed following the addition of a soluble nuclear fraction. It has also been shown that nascent pre-mRNA is specifically enriched in nuclear matrix preparations (Jackson et al., 1993; Wansink et al., 1993). Despite the many essential functions postulated for the nuclear matrix, few proteins involved in these processes have been characterized so far. Nuclear-mitotic apparatus protein (NuMA), a component of the nuclear matrix that also participates in spindle formation during mitosis, has been shown to localize to the nuclear speckled domains and to associate with snRNPs and with splicing complexes reconstituted in vitro (Zeng et al., 1994a,b). This suggests that NuMA is involved in binding the splicing machinery to the nuclear matrix.

The Ser-Arg (SR) family of protein splicing factors, which includes SC35 protein, contain a COOH-terminal domain that is rich in serine-arginine diamino acid repeats (Zahler et al., 1992). Many SR proteins share a common phosphoepitope recognized by mAb104 (Roth et al., 1989, 1991). Using antibodies against nuclear matrix antigens as well as the mAb104, recent studies have identified a class of nuclear matrix proteins which preferentially associate with exon-containing splicing complexes. These nuclear matrix proteins are related to the SR protein family and they could function in the recognition of exon sequences during the processing of pre-mRNA (Blencowe et al., 1994). Using the same type of assays, we observe that anti-4.1 antibodies are able to immunoprecipitate pre-mRNA, splicing intermediates and the reaction product, suggesting an association between components of the splicing machinery and protein 4.1. Multiple structural isoforms of 4.1 are produced by alternative splicing of the transcript of a single gene both in erythroid and nonerythroid tissues (Conboy, 1993; Tang et al., 1988, 1990). At least 10 exons can be selectively included or excluded from the mRNA transcript, in various combinations, to produce these isoforms. None of the 4.1 isoforms contain SR domains, therefore protein 4.1 does not appear to be a member of the SR family.

Most of the previous studies characterizing nuclear protein 4.1 were performed by immunodetection assays using different anti-4.1 antibodies (Correas, 1991; De Cárcer et al., 1995; Lallena and Correas, 1997; Krauss et al., 1997). There was still some scepticism to viewing protein 4.1 as a nuclear component. The isolation of a 4.1 cDNA clone encoding the isoform designated 4.1E has allowed us to show that the naturally-occurring protein 4.1E is targeted to the nucleus and that it associates with the splicing factor U2AF35. In addition, overexpression of protein 4.1E induces redistribution of endogenous SC35 from speckles to nuclear domains enriched in protein 4.1E. This effect is similar to that described for U1 snRNP 70K in overexpression experiments (Romac and Keene, 1995). These data support the view that protein 4.1 is a component of the nucleus involved in associations with splicing factors. Isoform 4.1E has a predicted size of 78,563 Da and it migrates on SDS-PAGE in the range of ~75-80 kDa, which coincides with that of the faster migrating 4.1 band present in HeLa nuclear extracts and that of the nuclear matrix protein 4.1p75 observed by De Cárcer et al. (1995). A 4.1 composite cDNA, encoding a protein which also runs in the range of ~75-80 kDa and with an exonic composition different from that of 4.1E, has been recently shown to localize in the nucleus (Krauss et al., 1997).

The study of the composition and organization of nuclear speckles is important not only to understand the structural organization of the nucleus, but also the location and regulation of pre-mRNA splicing in vivo. At present, it is not known what retains snRNPs and non-snRNP splicing factors in these structures, what is the structural organization of these components within these networks, and what are the mechanisms that allow their mobilization to sites of active transcription, for example upon adenovirus infection. It is therefore particularly valuable to identify components of these structures which have features suggestive of structural functions. NuMA, for example, localizes to speckles and contains protein domains with similarities to myosins and intermediate filaments (Lyderson and Pettijohn, 1980; Yang et al., 1992, Zeng et al., 1994b). In this report, we show that components of a family of proteins that integrate a bona fide structural network colocalize and associate with splicing factors. We think that the functional links between 4.1 and splicing described in this paper reflect an architectural role of 4.1 in the nucleus and the association of splicing factors with the 4.1 network. We do not believe that 4.1 has a direct role in the splicing process because 4.1 has none of the structural features described for the ~30 known proteins involved in pre-mRNA processing, including RNA binding motifs, arginine-serine-rich domains, helicase-like domains, etc. It seems extremely unlikely that 4.1 harbors completely unknown activities in the intensely studied biochemical apparatus for RNA processing, while the alternative hypothesis that 4.1 associates with splicing factors for structural purposes related to nuclear organization is not only consistent with the data of this work, but is also consistent with the well characterized functions of 4.1 as a component of skeletal networks.

In summary, colocalization, coimmunoprecipitation and in vitro splicing assays in immunodepleted extracts suggest that 4.1 antigens associate with factors involved in pre-mRNA splicing. The cloning of the 4.1E cDNA further demonstrates that the nuclear protein 4.1E binds to the splicing factor U2AF35 and that overexpression of 4.1E induces the redistribution of SC35, indicating that these interactions are functionally relevant and that they are important to understand the function of RNA processing factors in vivo.

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