Multiple protein 4.1 isoforms are originated by alternative pre-mRNA splicing, differential use of two translation initiation sites, and posttranslational modifications. The complexity of alternative splicing events suffered by the 4.1 pre-mRNA makes necessary the direct cloning of 4.1 full-coding cDNA sequences to ensure that the encoded 4.1 proteins are naturally occurring isoforms. We have approached this point by reverse transcription-polymerase chain reaction techniques using RNA from the nucleated human Molt-4 T-cell line as a starting template. Molecular cloning of 4.1 cDNAs using the second translation initiation codon has allowed us to identify two 4.1 isoforms, designated 4.1H and 4.1I, which are differentially targeted to the nucleus (4.1H) and the cytoplasm (4.1I). These two isoforms differ only in the inclusion (4.1H) or exclusion (4.1I) of 21 amino acids encoded by exon 16. A cluster of basic amino acids, KKKR, generated by joining of the sequences encoded by the constitutive exon 13 and the alternative exon 16, is necessary for the nuclear targeting of 4.1H, as demonstrated by site-directed mutagenesis analysis. Immunofluorescence microscopy and biochemical studies indicate that 4.1H belongs to the group of nuclear 4.1 proteins that are distributed diffusely throughout the nucleoplasm and that are extractable in 0.5% Triton X-100. This is the first demonstration of differential nuclear targeting by the presence of an alternative domain, among naturally occurring protein 4.1 isoforms.

Interactions between integral membrane proteins and the underlying spectrin-actin cytoskeleton play important roles in determining cell morphology, motility, activation, proliferation, contact, and even maintenance of specialized membrane domains. Protein 4.1 was initially identified as an 80-kDa multifunctional protein of the membrane-skeleton of human red blood cells. The function of 4.1 in this cell type as an stabilizing protein of the spectrin-actin network anchored to the plasma membrane via interactions with transmembrane proteins has been widely analyzed (for a review, see Ref. 1). The formation of the spectrin-actin-4.1 ternary complex is essential for the maintenance of normal red blood cell shape because alterations in the spectrin-actin binding site of protein 4.1, located at the C-terminal region of the molecule (2–5), are associated with congenital hemolytic anemias (6).

More recent studies have shown that avian erythrocytes and mammalian nonerythroid tissues present a variety of immunoreactive 4.1 polypeptides of Mr ranging from 30,000 to 210,000 (7, 8). Alternative splicing of the 4.1 pre-mRNA, the existence of two possible translation initiation sites, and posttranslational modifications are the causes for the generation of the extensive repertoire of 4.1 isoforms (1). Protein 4.1 expression has been most extensively studied in the erythroid system, where alternative splicing events involving exons 2 and 16 result in alterations in the structure of the N-terminal domain and the function of the spectrin-actin binding domain, respectively (9–15). In differentiated epithelial cells, an alternative splicing event regulates the expression of exon 17B, whereas exon 17A is specifically expressed in muscle cells (16, 17). All of these experiments show that the inclusion/exclusion of some 4.1 exons appears to be tissue-specific and developmentally regulated.

In mature red blood cells, protein 4.1 is localized at the periphery of the cell. In addition to this distribution, 4.1 presents different intracellular localizations in nonerythroid cells. This includes the cytoplasmic compartment (18, 19) and the nucleus (20–23). In the nucleus, several immunoreactive 4.1 proteins have been identified (20–23). Immunofluorescence microscopy and cell fractionation studies suggest that there are distinct nuclear 4.1 populations, as deduced by their differential responses to extraction with a variety of agents (21). Different isoforms of protein 4.1 are present in nuclear matrix preparations of several mammalian cells, suggesting that they could be structural elements of this network (21, 23). Furthermore, isolated erythrocyd 4.1 interacts with tubulin (24) and myosin (25), and a recent study also shows that protein 4.1 rearranges to the mitotic spindle during cell division (23). All of these data suggest that protein 4.1 may play additional roles different from the membrane-skeletal linking function assigned to erythroid 4.1.

Most of the studies analyzing 4.1 distribution in nonerythroid nucleated cells have been developed using anti-4.1 antibodies as tools. Therefore, it is not yet known whether the immunostaining of different subcellular compartments is due to the existence of different 4.1 isoforms or whether, on the contrary, the same 4.1 protein is targeted to different compartments by regulation through posttranslational modifications. The complexity of alternative splicing events suffered by the 4.1 pre-mRNA makes necessary the cloning of each of the different 4.1 coding sequences in a single piece to ensure that the encoded products correspond to naturally occurring 4.1 isoforms. In this study, we have approached this point by reverse transcription PCR techniques using mRNA from the...
nucleated human Molt-4 T-cell line. Molecular cloning of 4.1 isoforms has provided us the opportunity to analyze the intracellular localization of two isoforms that differ only in the inclusion or exclusion of exon 16. Here, we report that a 4.1 isoform containing the sequences encoded by exon 16, named 4.1H, is differentially targeted to the nucleus, whereas a 4.1 isoform lacking those sequences, named 4.1I, is localized in the cytoplasm. A cluster of basic amino acids (KKKK) generated by joining exon 13 and 16 sequences was identified as necessary for the nuclear targeting of 4.1H by mutagenesis analysis. This nuclear isoform belongs to the group of 4.1 proteins extractable in Triton X-100 and with a diffuse distribution in the nucleoplasm. This is the first demonstration of differential nuclear targeting by the inclusion of an alternative domain among naturally occurring protein 4.1 isoforms.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**Human T lymphoid Molt-4 cells were used as RNA source for cDNA cloning, fibroblastic COS-7 cells from monkey kidney and HeLa cells were used for transient expression experiments, and COS-7 and Madin-Darby canine kidney (MDCK) cells for immunofluorescence and Western blot analysis. Molt-4 cells were grown on tissue culture flasks in RPMI 1640 medium (Life Technologies, Inc.). COS-7, HeLa, and MDCK cells were grown on culture dishes or on glass coverslips in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.). Both media were supplemented with 1% glutamine, 5% (v/v) fetal calf serum (Life Technologies, Inc.), penicillin (50 units/ml), and streptomycin (50 units/ml). Cultures were maintained at 37 °C under a 5% CO2/95% air humidified atmosphere. Transfection experiments were performed by electroporation using the Electro Cell Manipulator 600 (BTX, San Diego, CA), using the following parameters: 200 V, 950 microfarads, and 480 ohms in a 4-mm-gap cuvette. Cells were processed 48 h after transfection.

**RNA Extraction and cDNA Cloning—**Clotryplastic RNA was extracted, as described previously (26). 4.1 mRNA isoforms were specifically reverse-transcribed using AMV reverse transcriptase (Promega) and the specific 4.1 antisense primer cDNA2-E22a (CT-GAATTCTGACTATGGCAGGTTAGC), corresponding to 3′-untranslated sequences contained in exon 22 (Isogen Bioscience BV, Maarssen, The Netherlands). The 4.1 cDNAs were then PCR amplified under the conditions described by Conboy et al. (11) using Taq polymerase (Promega). A c-Myc antisense primer complementary to the 3′-end of the 4.1 coding sequence (c-Myc, 9E10 epitope (MEQKLIEL), corresponding to the 3′-end of the 4.1 coding sequence). Reaction products were cloned into the pMOSBlue T-vector (Amersham Pharmacia Biotech). For expression experiments, 4.1 cDNAs were tagged at their 3′-termini by PCR. Reactions were carried out using sinE2 and stop-c-Myc, an antisense primer complementary to the 3′-end of the 4.1 coding sequence and encoding the c-Myc 9E10 epitope (MEQKLIEL-SEEDL) (27). The cDNAs encoding epitope-tagged 4.1 proteins were finally cloned into the pSRa mammalian expression vector (28).

**Antibodies—**Anti-4.1 antibodies designated 10a and 10b are affinity-purified polyclonal antibodies against synthetic peptides designed according to the sequence of the 8–10-kDa 4.1 domain, involved in the association of 4.1 with spectrin and actin (3). Antibodies 10a and 10b recognize epitopes encoded by the alternative exon 16 and the constitutive exon 17, respectively (3). Anti-c-Myc monoclonal antibody 9E10 (27) was obtained from the American Type Culture Collection. Goat anti-rabbit IgG and anti-mouse IgG secondary antibodies, conjugated with horseradish peroxidase, fluorescein isothiocyanate, or Texas Red isothiocyanate were obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL).

**Immunofluorescence Microscopy—**Cells grown on glass coverslips were fixed in 10% formalin (Sigma) for 20 min at room temperature, and the unreacted formaldehyde was unreacted by incubation with 10 mM glycine for 10 min. Cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and blocked by incubation with 3% bovine serum albumin. Cells were incubated with the appropriate antibodies and processed as described by De Cárdenas et al. (21). DNA was stained with 1 μg/ml 4′,6′-diamidino-2-phenylindole (Sigma) for 5 min at room temperature. After incubation, preparations were washed, mounted on microscope slides, and examined with a Zeiss epifluorescence microscope. Controls to assess the specificity and the lack of cross-labeling included incubations with nonimmune rabbit serum and irrelevant monoclonal antibodies or omission of either of the primary antibodies. In transfection experiments, anti-4.1 antibodies were used 25-fold diluted, relative to concentrations used for detection of endogenous protein, in order to detect only exogenous epitope-tagged 4.1 proteins. Mock-transfected cell controls were analyzed with anti-c-Myc or anti-4.1 antibodies.

**Sequencing—**Sequencing of 4.1 cDNAs was done using the ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase, FS (Perkin-Elmer). Sequencing PCR products were carried out in a GeneAmp PCR System 9600 (Perkin-Elmer) and examined in an ABI 373A automatic sequencer. In order to verify the entire cloned sequences, reactions were carried out from different cDNAs, using 4.1 specific primers designed according to the sequences contained in exon 16. The sequences were deposited in GenBank (11) under accession number M61733 (11).

**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 mPos-BLUE cloned 4.1 cDNAs as templates. Synthesized proteins were radiolabeled by including [35S]methionine (Amersham Pharmacia Biotech) in the reaction. After fractionation by SDS-PAGE in 8% acrylamide minigels, proteins were stained with Coomasie Blue, and the gels were dried and exposed on Kodak autoradiographic films.

**Mutagenesis and DNA Constructs—**Mutagenesis of the cluster of basic amino acids present in 4.1H was made by the overlap extension technique (29). To obtain a full-length 4.1H cDNA, primer cDNA2-E22a (TCACTCATCAGCAATCTCGG) (an antisense primer corresponding to the 3′-end of the 4.1 coding sequence). Reaction products were cloned into the pCRIII mammalian expression vector (Invitrogen BV, Leek, The Netherlands). Additionally, a 4.1β-galactosidase chimeric cDNA was constructed using two complementary oligonucleotides that were annealed and cloned into the KpnI site of pCH110 plasmid (30) (GenBank accession number U13845). The construct encodes a fusion protein that contains 20 residues from 4.1H, including the cluster of basic amino acids, at the N-terminal region of β-galactosidase.

**Protein Extractions and Western Blot Analysis—**Cells grown on petri dishes were rinsed twice in PBS and then either lysed in 100 μl of solubilizing Laemmli buffer (31) or subjected to extraction with 0.5% Triton X-100, as described previously (21). Protein fractions were processed, separated by SDS-PAGE and transferred to Immobilon-polyvinylidene difluoride (Millipore, Bedford, MA) in CAPS buffer, pH 11. Membranes were processed and developed as described previously (21).

**RESULTS**

**Cloning of Lymphoid 4.1 cDNAs—**Previous studies have shown a great diversity of 4.1 isoforms that are partly generated by alternative splicing of 4.1 pre-mRNA. A summary of alternative and constitutive exons is shown in Fig. 1A. The prototypical erythroid protein 4.1 is translated from a translation initiation site present in exon 4 (ATG-2). Higher molecular weight 4.1 isoforms can be generated when a complex alternative splicing event takes place that consists of the inclusion of exon 2′ concomitantly with the exclusion of exon 3. This event introduces an in-frame translation initiation site (ATG-1) present in exon 2′, which adds 209 amino acids N-terminal to the prototypical erythroid 4.1 protein. Thus, two different 4.1 isoform groups can be generated attending to whether ATG-2 or ATG-1 is used as the translation initiation site. These two groups are denominated low molecular weight (LMW) and high molecular weight 4.1 isoforms, respectively (Fig. 1B).

To clone directly in a single DNA fragment the coding sequence corresponding to the subset of low molecular weight 4.1 isoforms, we adopted a reverse transcription PCR approach, using RNA from the Molt-4 T-cell line as the starting template and the 4.1 oligonucleotide primers specific for this subset of cDNAs. 4.1 cDNA was synthesized using the oligonucleotide primer cDNA2-E22a corresponding to the 3′-untranslated sequences contained in exon 22. The cDNA was then amplified by PCR using the oligonucleotides sinE2 (a 5′ sense amplification primer that excludes exon 2′ and matches the joining of exons 1 and 2) and stopE21a (an antisense primer corresponding to the sequence of exon 3).

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the 3′-end of the 4.1 coding sequence) (Fig. 1C). Thus, the resulting cDNAs encode proteins belonging to the group of LMW 4.1 isoforms.

**Identification of Two 4.1 cDNAs Differing in the Presence or Absence of Exon 16 and Displaying Different Intracellular Localization**—The 4.1 PCR products were cloned into the plasmid pMOS-BLUE and sequenced. Seven different low molecular weight 4.1 cDNAs were identified on the basis of the presence or absence of specific exons. In this study, we focused on the analysis of two of them, designated 4.1H and 4.1I, differing only in the presence (4.1H) or absence (4.1I) of exon 16 sequences. A diagram showing the exon composition of 4.1H and 4.1I cDNAs is presented in Fig. 1D.

The 4.1H and 4.1I cDNAs were tagged with sequences encoding the c-Myc 9E10 epitope 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 4.1H or 4.1I were analyzed 48 h after transfection by double-labeling immunofluorescence microscopy using anti-c-Myc (9E10) and anti-4.1 (either 10a or 10b) antibodies.

As detected by anti-c-Myc antibody, the distribution of the 4.1H isoform was mainly diffuse in the nucleoplasm, avoiding nucleoli (Fig. 2A), whereas the 4.1I isoform was localized in the cytoplasm, drawing bright fibers along cellular prolongations (Fig. 2D). Over 20 transfection experiments were performed and analyzed. As for 4.1H, ~72% out of more than 400 analyzed transfected cells had the staining pattern shown in Fig. 2A, whereas the remaining 28% displayed intense cytoplasmic and nuclear staining. In case of 4.1I expression, the pattern shown in Fig. 2D is representative of 71% out of more than 400 transfected cells analyzed. The remaining 29% of the cells displayed cytoplasmic and nuclear staining. Similar distribution patterns were observed for 4.1H and 4.1I using the anti-10b antibody (data not shown). Cells transfected with vector lacking 4.1cDNA insert showed no staining with either anti-c-Myc or anti-4.1 antibodies (not shown).

Exon 16 is another alternative exon (9); its inclusion in mature mRNA has been evolutionarily conserved (15) and is developmentally regulated in erythroid cells (9, 14, 15), helping to provide mechanical stability to red blood cells (13). The selective presence of exon 16 sequences in the 4.1H cDNA led us to use the anti-4.1 antibody (10a), raised against a synthetic peptide included in the sequence encoded by exon 16 (3), to demonstrate the expression of exon 16 in the 4.1H isoform. Thus, cells transfected with 4.1I showed the characteristic cytoplasmic staining pattern with the anti-c-Myc antibody (Fig. 2D) but were not immunostained with the anti-10a antibody (Fig. 2F). By contrast, cells transfected with 4.1H cDNA were immunostained with both anti-c-Myc (Fig. 2A) and anti-10a (Fig. 2C) antibodies. Similar results were obtained in transiently transfected HeLa cells (not shown). These experiments demonstrate...
expression of exon 16 in 4.1H and not in 4.1I.

Characterization of the Proteins Encoded by the 4.1H and 4.1I cDNAs—The apparent molecular mass of the in vitro (Fig. 3A) or in vivo (Fig. 3B) synthesized 4.1H and 4.1I proteins were determined by SDS-PAGE. 4.1H and 4.1I cDNAs were transcribed in vitro with T7 RNA polymerase and translated in vitro using a rabbit reticulocyte lysate. This yielded products of apparent molecular mass of ~82 kDa for 4.1H (Fig. 3A, lane 1) and of ~80 kDa for 4.1I (Fig. 3A, lane 2). Similar sizes were observed for both isofoms in extracts prepared from transiently transfected COS cells (Fig. 3B). Antibodies to the c-Myc tag or to 4.1 (anti-10a) recognized both 4.1H and 4.1I isoforms (Fig. 3B, lanes 1–6), whereas antibody 10a recognized only the 4.1H isoform (Fig. 3B, lanes 7–9).

Expression of Endogenous 4.1 Proteins Containing Exon 16-encoded Sequences—Inclusion of exon 16 sequences in 4.1 mRNA is a late event in erythropoiesis (11, 14), and its presence has been reported in many nonerythroid cell lines and tissues. Hence, mRNAs and partial cDNA clones containing exon 16 have been obtained from mouse brain, embryos, endothelial cells, spleen, skeletal muscle, and testis and from human endothelial cells, fetal liver, bone marrow, brain, liver, and intestine (10, 11, 32). To further investigate the expression of exon 16-encoded sequences in endogenous 4.1, we carried out both immunoblot and immunofluorescence analysis with anti-10a antibody in nucleated COS-7 (Fig. 4A) and MDCK (Fig. 4B) cells. In interphase cells, anti-10a antibody gave a diffuse, mainly nuclear staining. In metaphase cells, the staining became cytoplasmic, excluding the chromosomal plate (Fig. 4B). Total cell extracts, analyzed by Western blotting with anti-10a, presented several immunoreactive bands in the range of ~40–170 kDa (Fig. 4C). Some of them are shared by COS and MDCK cells, whereas others seemed to be expressed by only one of the cell lines. Polypeptides with apparent molecular masses of ~80–85 kDa were detected in both samples, coinciding with the range in which 4.1H isoform runs.

Mutagenesis of a Basic Tetrapeptide Encoded by the Exon 13/16 Junction Prevents Nuclear Import of Protein 4.1H—Whereas small molecules, ions, and metabolites freely diffuse through the nuclear pore, import of molecules larger than 40 kDa requires energy and is mediated by signals on the import substrates (reviewed in Ref. 33). "Classical" nuclear localization sequences (NLSs) are generally characterized by one or more clusters of basic amino acids (33). Inspection of the 4.1H sequence revealed that a cluster of basic amino acids (KKKR) generated by the joining of exon 13 and 16 sequences is present in this isoform but not in 4.1I. The first lysine within the cluster corresponds to the last amino acid encoded by the constitutive exon 13, whereas the other basic residues are the first three amino acids encoded by the alternative exon 16. This cluster closely resembles the prototypical NLS of the SV40 large T-antigen. To determine whether this basic region is necessary for targeting 4.1H to the nucleus, a construct (4.1Hmut) was generated in which the basic amino acid cluster (KKKR) was substituted with uncharged amino acids (TNSG). 4.1Hmut was transiently expressed in COS-7 cells, and its localization was compared with that of the wild type 4.1H protein by immunofluorescence microscopy. The substitution of the basic amino acids (KKKR) in 4.1H drastically affected its distribution. Thus, whereas 4.1H is targeted to the nucleus (Fig. 5A), the mutant was excluded from this compartment and remained in the cytoplasm (Fig. 5B). This indicates that the basic tetrapeptide (KKKR) encoded by the exon 13/16 junction in the isoform 4.1H is necessary for its nuclear targeting.

The NLS of the SV40 large T-antigen was defined as a seven-amino acid sequence, sufficient to confer nuclear localization to a cytoplasmic protein (33). However, other sequences surrounding this heptapeptide were found to strongly affect its import rate, in particular, a phosphorylation site for casein kinase II, localized 13 amino acids N-terminal from the heptapeptide (34). To test the efficiency of the putative NLS present in 4.1H to target a cytoplasmic reporter to the nucleus, we fused the last six residues encoded by exon 13 and the first fourteen encoded by exon 16 to β-galactosidase. These 20 amino acids (PTEAWKKKERELEGNY11YR) include the basic tetrapeptide and also a tyrosine, eight amino acids C-terminal from the basic cluster, the phosphorylation state of which has been demonstrated to modulate actin-spectrin binding activity in vitro (35). Cells expressing β-galactosidase or the NLS-like sequence fused to β-galactosidase, analyzed 48 h after transfection, showed similar staining patterns, mostly cytoplasmic in both cases (not shown).

4.1H Belongs to a Nuclear 4.1 Population That Is Distributed Diffusely throughout the Nucleoplasm, Except for the Nucleoli, and Is Extractable in Triton X-100—Previous studies from our laboratory have shown that protein 4.1 is distributed in nuclear speckles enriched in splicing factors and is also localized in the space between the speckles, with the exclusion of nucleoli (20–22). In addition, cell treatments with different agents result in the extraction of different 4.1 polypeptide
bands, suggesting that some protein 4.1 isoforms are more tightly bound to nuclear domains than others. In this regard, we (21) and others (23) have detected 4.1 in nuclear matrices isolated from mammalian cells. Because the 4.1H isoform is distributed diffusely throughout the nucleoplasm of transfected cells, avoiding nucleoli (see Figs. 2 and 5), we decided to investigate whether it is tightly bound to the nucleus or not. Fig. 6A shows that the 4.1H isoform expressed in transfected cells (lane T) was totally extracted in 0.5% Triton X-100 (compare lane S with lane P). The behavior of endogenous 4.1 proteins was also examined in experiments carried out in parallel in untransfected cells (Fig. 6B). In agreement with previous studies (7–10, 20, 21, 23), several 4.1 polypeptides were detected in total cell extracts (Fig. 6B, lane T). The 4.1 population in the range of 80 kDa was mainly present in the Triton-soluble fraction (Fig. 6B, lane S). Protein 4.1 isoforms of different sizes that are partially soluble or insoluble in Triton are also observed in Fig. 6B, lanes S and P.

The same type of experiments were performed in situ using cells grown on coverslips. The intense and diffuse nuclear staining pattern observed in cells transfected with 4.1H and detected with the anti-10b antibody (Fig. 7A) was removed by Triton X-100 treatment (Fig. 7C). The punctate immunostaining pattern observed in Fig. 7C was caused by the Triton-insoluble endogenous 4.1 proteins because the same cells treated with the anti-c-Myc antibody did not present any staining (data not shown). Control experiments performed in untransfected cells showed both diffuse and punctate immunostaining patterns (Fig. 7B). The diffuse pattern was mainly extracted with Triton X-100, whereas the punctate pattern

FIG. 3. In vitro translation and in vivo expression of 4.1H and 4.1I isoforms. A, in vitro coupled transcription and translation of pMosBLUE cloned 4.1 cDNAs was performed as described in the text. Reaction products were labeled with [35S]methionine and autoradiographed.

Lane 1 (H), 4.1H; lane 2 (I), 4.1I; lane 3 (C), a control containing all components of the mixture except the template 4.1 cDNA. B, Western blot analysis of total extract proteins from 4.1H-transfected (H; lanes 1, 4, and 7), 4.1I-transfected (I; lanes 2, 5, and 8), or control untransfected (C; lanes 3, 6, and 9) COS cells. Immunodetection with anti-c-Myc (lanes 1–3), anti-10b (lanes 4–6), and anti-10a (lanes 7–9). Both in vitro and in vivo analyses show that 4.1H and 4.1I have apparent molecular masses of 82 and 80 kDa, respectively.

FIG. 4. Detection of endogenous 4.1 proteins containing exon 16-encoded sequences in COS and MDCK cells. Untransfected COS and MDCK cells were processed as indicated under “Experimental Procedures.” Immunofluorescence analysis was carried out using anti-10a antibody, rendering a diffuse, mainly nuclear staining both in COS (A) and MDCK (B) cells, avoiding nucleoli. Note that a mitotic cell presents cytoplasmic staining surrounding metaphasic chromosomes (B). C, total cell extracts were analyzed by Western blotting with anti-10a antibody. Polypeptides with apparent molecular masses of ~80–85 kDa are detected in both COS-7 and MDCK cell extracts.

FIG. 5. Subcellular localization of wild type and mutated 4.1H. COS cells were transfected with either 4.1H (A) or 4.1Hmut (B) cDNAs, labeled with anti-c-Myc antibody 48 h after transfection, and examined by epifluorescence microscopy. Protein 4.1H was targeted to the nucleus, whereas 4.1Hmut was not and remained in the cytoplasm. Bar, 10 μm.

FIG. 6. Isoform 4.1H is extracted in 0.5% Triton X-100. COS cells transfected with 4.1H cDNA (A) or untransfected cells (B) were treated as indicated under “Experimental Procedures.” Total lysates (T) and the Triton-soluble (S) and Triton-insoluble pellet (P) fractions were analyzed for the presence of 4.1H (A) or endogenous 4.1 proteins (B) by Western blot using anti-10b antibody. All immunoreactive 4.1H was in the soluble fraction (see lane S in A). Endogenous 4.1 with a molecular mass similar to that of 4.1H is mainly distributed in the Triton-soluble fraction (see lane S in B). In order to achieve visualization of endogenous 4.1 proteins, panel B was overexposed relative to panel A.
The expression of exon 16 has been extensively analyzed in the erythroid system. This exon is regulated by alternative splicing, being excluded from erythroid precursors and included in mature red cells. Many nonerythroid cells and tissues also include this alternative exon, as shown by mRNA and partial cDNA clone analysis (10, 11, 32). Inclusion of exon 16 generates protein 4.1 isoforms having a fully functional spectrin-actin binding domain and contributes to the mechanical stabilization of the plasma membrane in mature erythrocytes (9, 13–15). Here, we show that endogenous 4.1 proteins containing exon 16 sequences are present in nucleated nonerythroid cells, as detected with anti-10a antibody in immunofluorescence and Western blot analysis. Our data also indicate that lymphoid cells contain the isoform 4.1H, which includes exon 16 next to exon 13, thus resulting in the generation of a cluster of basic amino acids that enables nuclear targeting of 4.1H, instead of favoring its binding to actin filaments in the cytoplasm.

The first sequence reported for protein 4.1 (38) corresponded to a human reticulocyte LMW 4.1 isoform of 588 amino acids originally considered as the prototypical erythroid 80-kDa protein (Prosite accession number PS00660). The second sequence reported (9) corresponded to a lymphoid LMW 4.1 isoform of 566 amino acids (GenBank™ accession number J03796). The third one corresponded to a LMW 4.1 protein of 641 amino acids (GenBank™ accession number M61733). These three 4.1 sequences are composite amino acid sequences. The complex pathway of splicing events observed in the 4.1 pre-mRNA indicates that many 4.1 isoforms can be expected to exist in the cell. The two isoforms described in this study have been each isolated in a single piece and contain 4.1 full-coding sequences for 622 amino acids (4.1H) and for 601 amino acids (4.1I).

We (21) and others (23) have shown that a 4.1 protein of approximately 75 kDa is a component of the nuclear matrix of mammalian cells. Protein 4.1H is not present in nuclear matrices prepared from cells transfected with the 4.1H cDNA. These data reinforce previous observations (20–23) indicating that there are several different types of protein 4.1 in the nucleus. The role that 4.1H may be playing within the nucleus is intriguing. Because 4.1H contains the complete spectrin-actin binding site present in exons 17 and 16 and because the existence of nuclear actin (39–41) and spectrin (42) has been reported, one possibility is that 4.1H could be forming a ternary complex with these two nuclear proteins, as its erythroid counterpart does in the cytoplasm. Other potential partners for 4.1H, such as myosin (41, 43) and calmodulin (42), have also been detected in the nucleus.

In mammalian cells, pre-mRNA splicing proteins are mainly accumulated in two domains: “speckles,” of which 20–50 exist per nucleus, and “coiled bodies,” of which 1–5 exist per nucleus (reviewed in Refs. 44 and 45). Some splicing proteins also are distributed diffusely throughout the nucleoplasm, with the exclusion of the nucleolus. The majority of pre-mRNA splicing factors organized in nuclear speckles are resistant to Triton X-100 extraction, but the more diffusely distributed pool of splicing factors, including SF2/ASF, U2-B*, and the Sm antigen, is largely soluble (46). Previous studies from our laboratory have shown that endogenous 4.1 is distributed diffusely throughout the nucleoplasm, excluding nucleoli, as well as in speckles enriched in splicing factors (20–22). Thus, splicing
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factors might also be other potential partners for nuclear protein 4.1 isoforms.

The cloning of the lymphoid 4.1H isoform and its identification as a nuclear component of the diffusely distributed pool of nuclear 4.1 proteins opens up future investigation into the role that protein 4.1 plays in this cellular compartment.

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