RELATION WITH SPlicing AND EARLY HEAT SHOCK-INDUCED SPlicing ARREST* 

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Using antibody 2H9 from our heterogeneous nuclear ribonucleoproteins (anti-hnRNP) monoclonal antibody library, we previously showed in HeLa cells that a 35–37-kDa protein doublet switches from the hnRNP complexes to the nuclear matrix following a 10-min heat shock at 45 °C (1 Lutz, Y., Jacob, M., and Fuchs, J. P. (1988) Exp. Cell Res. 175, 109–124). cDNA cloning and sequencing revealed an hnRNP protein (2H9) which is a new member of the hnRNP F, H/H' family. Protein 2H9 displays two consensus sequence-type RNA binding domains (CS-RBD) showing 80–90% homology with two of the three CS-RBDs of hnRNP F and H/H'. Another common feature is the presence of two glycine/tyrosine-rich auxiliary domains located at the C terminus and between the two CS-RBDs. At the functional level we show that specific anti-2H9 peptide antibodies can directly inhibit an in vitro splicing system. Moreover, the 2H9 protein doublet is no more present in nuclear extracts from such briefly stressed cells, which interestingly correlates with the inability of these extracts to catalyze in vitro splicing reactions. Taken together, our data suggest that these proteins are involved in the splicing process and also participate in early heat shock-induced splicing arrest by transiently leaving the hnRNP complexes. These 2H9 proteins, which are encoded by a single gene located on human chromosome 10, were also found to be associated with nuclear bodies in situ.

Most RNA polymerase II transcripts or pre-mRNAs undergo a series of post-translational processing events leading to mRNAs that are further exported to the cytoplasm. This results in the simultaneous presence within the nucleus of a large variety of pre-mRNAs, mRNAs, and intermediate reaction products, which are collectively termed heterogeneous nuclear RNAs (hnRNAs). As soon as they emerge from the transcription complex and throughout maturation, hnRNAs interact with hnRNP proteins (hnRNPs) and snRNPs; these assemblies, also called hnRNP complexes, serve as the substrate for RNA processing events, including splicing (2–5). Ultrastructural investigations in fact support the idea that the in situ form of these hnRNP complexes are the intermediate and perichromatin fibrils (6–8). It is also thought that these complexes interact with the nuclear matrix which might be involved itself in splicing (9–14). Among the numerous hnRNPs proteins (2, 15) several have been cloned and sequenced, but there is nevertheless only limited information available on their function, except for a few of them (2, 16). To investigate the structure and the function of the hnRNP proteins, we previously raised a monoclonal antibody library directed against hnRNP complexes purified from HeLa cells (1).

Among other aspects we used our anti-hnRNP monoclonal antibody library to investigate heat shock-induced splicing arrest. Indeed, splicing was previously shown to be transiently shut down following heat shock at the upper range of the stress response (17, 18). Since such a stress does not modify the general characteristics of hnRNP complexes (1, 19, 20), we assumed that splicing inhibition might be linked to changes at the level of a limited number of individual proteins. To check this hypothesis we screened our antibody library by indirect immunocytofluorescence on normal and heat-shocked HeLa cells, so as to detect possible differences either in signal intensity or distribution between both types of cells. As early as 10 min after the onset of a heat shock at 45 °C, we actually showed that the structure of hnRNP complexes is altered in a very subtle and reversible way. Indeed, among all antibodies from our library, only two of them revealed the kind of differences we were looking for. These antibodies named 2H9 (1) and 6D12 (20) recognize a 35–37- and a 72.5–74-kDa protein doublet, respectively. In our immunocytochemical assay using fixed cells, these antigens were only available to the antibodies in stressed cells, although Western blot analyses showed no quantitative variation in stressed versus normal cells. In stressed cells these antibodies revealed a bright nuclear signal present within the interchromatin space as numerous small dots. This signal persists for 2 h after cells were taken back at 37 °C and then gradually decreases and finally disappears after 6 h, which again corresponds to the situation in normal cells. By combining immunological and subcellular fractionation techniques, we could relate this observation to a rapid switch of both protein doublets from the hnRNP complexes to the nuclear matrix.
matrix, a structure to which they bind very strongly (1, 20).

In this paper, we describe the cloning and the sequencing of the 2H9 cDNA that encodes the 35–37-kDa (or 2H9) proteins from HeLa cells. The deduced amino acid sequence shows that they are genuine hnRNPs proteins that are related to the hnRNP F and H/H' family. In order to check in a direct way whether or not the 2H9 proteins are required for pre-mRNA splicing, we tested the effect of anti-2H9 antibodies on an in vitro cell-free system that faithfully splices E1A transcripts of adenovirus 2 (21). Furthermore, since stressing HeLa cells for 10 min at 45° C causes nuclear extracts to be inactive in in vitro splicing (22), such extracts were also analyzed and compared with normal extracts, so as to find out whether there is a correlation between the status of these 2H9 proteins in the cell-free system and the fact that within the cell they transiently leave the hnRNP complexes (1).

**EXPERIMENTAL PROCEDURES**

**Monoclonal Antibody 2H9 and Western Blot Analysis—**Monoclonal antibody 2H9 (IgG\(_1\)), which is specific for the 2H9 proteins, was obtained as described previously (1), after immunizing Balb/c mice with hnRNP complexes purified from HeLa S3 cells. Both hybridoma culture supernatant and ascites fluids were used as monomasonic antibody sources.

For Western blotting analysis, proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose sheets, the blots being further probed with 2H9 supernatant and 125-I-labeled anti-mouse Ig-F(ab\(_{2}\)) (1).

**Peptidic Maps—**Peptidic maps were as described previously (20).

Briefly, 2H9 proteins were immunoprecipitated from a nuclear extract, resolved by SDS-PAGE, and the 35- and 37-kDa bands cut out. Polypeptides were radioiodinated (Na\(^{125}\)I) within the gel, using the chloramine-T method, and further hydrolyzed with trypsin or thermolysin. Peptides were resolved in two dimensions on cellulose-coated thin layer chromatography plates and visualized by autoradiography.

cDNA Libraries, cDNA Cloning, and Sequencing—cDNA cloning was by using an Agt11- and a Agt10-cDNA library. The Agt11 library comprises prNone-primed cDNAs cloned into the EcoRI site of the expression vector; template RNA was a poly(\(A^+)\) fraction isolated from human MCF-7 cells. The Agt10 library we constructed according to Sambrook et al. (23) contains oligo(dT)-primed cDNAs cloned into the EcoRI site of the vector; template RNA was a poly(\(A^+)\) fraction isolated from HeLa S3-cell cytoplasm and purified on poly(U)-Sepharose.

cDNA cloning was essentially performed by using standard techniques described by Sambrook et al. (23). The random-primed Agt11 library was immunoscreened for cDNA fragments encoding the epitope recognized by monoclonal antibody 2H9. Such fragments were then used to screen the oligo(dT)-primed Agt10 library, in an attempt to isolate full-size cDNAs. For sequencing into plasmid SK+ and SK-3, method A was used. After the generation of partially overlapping deletion mutants, the nucleotide sequence of each cDNA strand was determined according to the chain termination method (24), using a single primer corresponding to a vector sequence. In some cases primers corresponding to cDNA sequences were used to obtain additional sequence information.

**Northern Blotting—**Purified cytoplasmic poly(A\(^+)\) RNA was analyzed by electrophoresis on 1.2% agarose, 6% formaldehyde gels and transferred to nitrocellulose sheets by capillary blotting with 20 × SSC. Prehybridization and further hybridization with 32P-labeled probes were carried out according to Sambrook et al. (23).

**In Vitro Expression and Immunoprecipitation—**Recombinant plBluescript was linearized and RNA transcribed in both directions with T3 and T7 RNA polymerase in the presence of m\(^{7}\)G(5\(^'\))pppG. *In vitro* translation in the presence of rabbit reticulocyte lysates (Amersham Corp.) and \[^{35}\]S)methionine was performed according to the manufacturer. Specific translation products were immunoprecipitated with monoclonal antibody 2H9 previously coupled to CNBr-activated Sepha-rose. Proteins were resolved on an SDS-10% polyacrylamide gel and the labeled proteins visualized by fluorography, immunoprecipitated monoclonal antibody (1 \(\mu\)g/ml) and rabbit anti-p80 coilin antibody (1:1000) in PBS. The cells were then washed with PBS/Triton/Na\(_2\) and further incubated for 45 min with a mixture of Cy3-conjugated goat anti-mouse Ig (1:200) and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:100, Jackson Immunoresearch Lab.) in PBS/Triton/Na\(_2\). The cells were washed again and DNA counterstained with Hoechst 33258.

**Monoclonal Antibody 2H9 and Western Blot Analysis—**Potentially immunogenic amino acid stretches of the 2H9 protein sequence were determined from the hydrophilicity (25) and mobility (26) plots drawn from the predicted primary structure. Synthetic peptides with an additional Cys residue were prepared and further coupled to ovalbumin activated with m-maleimidobenzoyl-Nsuccinimide ester. For polyclonal antibodies, New Zealand rabbits were injected at 2-week intervals with 200 \(\mu\)g of peptide-ovalbumin. Serum were tested by immuno blotting for their ability to react with the 2H9 proteins, and antibodies were further immunopurified on a Sulfolinik column (Pierce) coupled to the relevant peptide. For monoclonal anti-peptide antibodies, Balb/c mice were injected at 2-week intervals with 50 \(\mu\)g of peptide-ovalbumin, until specific humoral antibodies were detected. The mice were then given three 10-\(\mu\)g boosts over 3 days. On day 4, splenic cells were fused with X-63 myeloma cells; hybridoma cell lines were established as described previously (1). Monoclonal antibodies were purified from as ctes liquids on ABx columns (J. T. Baker).

**Cells and Heat Shock, Cytoplasmic and Nuclear Extracts—**HeLa S3 cells were grown in Eagle's suspension medium supplemented with 20% newborn calf serum and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 \(\mu\)g/ml). For heat treatment, cells were concentrated to 5 × 10\(^6\) cells/ml and transferred to culture flasks, which were then immersed in a water bath for 10 min at 45° C. Cytoplasmic S100 and nuclear ex tracts were prepared according to Dignam et al. (27) and dialyzed against buffer D for splicing assays.

**Antibody Effect on In Vitro Splicing—**Plasmid Sp4 contains the natural E1A sequence (nucleotides 533-1342) of adenovirus 2 between the Smal and Xba sites of the polylinker of vector pSP65 (21). Capped E1A-precursor RNA was synthesized using SP6 RNA polymerase in the presence of m\(^{7}\)G(5\(^'\))pppG and \((\alpha-32P)CTP\), and further purified as de scribed previously (21). Splicing reactions were carried out for 2 h at 30 °C in 25 \(\mu\)l of reaction mixture. Mixtures containing 10\(^6\) cpm of [\(^{32}\]P]pre-mRNA (4. ng), 10 \(\mu\)l of nuclear extract (6–8 mg of protein/ml) in the presence of 2.6 mM MgCl\(_2\), and 60 mM KCl as described previously (21). For studying the direct effect of antibody on splicing, the nuclear extract was preincubated at 0 °C for 30 min with either 10 \(\mu\)g of purified monoclonal antibody or 6 \(\mu\)g of purified polyclonal antibody. For control, the nuclear extract was preincubated with either an equal amount of the same antibody previously heat-denatured at 55°C for 10 min or pretreated with 10 \(\mu\)M DTT at 30 °C for 30 min, or with nonrelevant antibody. After phenol extraction and purification, RNA was analyzed on 5.2% polyacrylamide gels in \(8 \times\) urea.

**Double Immunocytofluorescence and Confocal Microscopy—**Schaffner HeLa cells (10\(^5\) cells/ml) in Dulbecco's modified Eagle me dium supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 \(\mu\)g/ml) were grown on glass slides for 3 days, washed with PBS, and fixed for 4 min with 2% paraformalde hyde in PBS. When investigating the effect of heat shock, cell cultures were immersed in a water bath for 10 min at 45 °C prior to fixation. The cells were then permeabilized for 20 min with 0.1% Triton X-100, 0.05% Na\(_2\) in PBS. After several washings with PBS/Na\(_2\), they were incubated overnight with a mixture of anti-His-tagged monoclonal antibody (1 \(\mu\)g/ml) and rabbit anti-p80 coilin antibody (1:1000) in PBS. The cells were then washed with PBS/Triton/Na\(_2\) and further incubated for 45 min with a mixture of Cy3-conjugated goat anti-mouse IgG (1:200) and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:100, Jackson Immunoresearch Lab.) in PBS/Triton/Na\(_2\). The cells were washed again and DNA counterstained with Hoechst 33258.
The preparations were mounted in glycerol/PBS (4:1) containing 5% propyl gallate as an anti-oxidant. Immunofluorescence images from 0.6-µm optical sections were obtained using a Leica confocal laser scanning microscope equipped with a PL APO 100/1.4 oil immersion lens and a krypton-argon laser. Excitation wavelengths of 488 and 568 nm were selected for fluorescein isothiocyanate and Cy3, respectively. Hoechst images were acquired using a Kappa video camera connected to the Leica digitizer. Pseudo-colored images of the signals were generated and further superimposed using an image processing program.

**RESULTS**

**Isolation of cDNA Encoding the 2H9 Proteins**—As described before by Lutz et al. (1), the 2H9 proteins (35 and 37 kDa) shown in Fig. 1 (left panel) share a series of characteristics, including a common epitope recognized by monoclonal antibody 2H9. In order to rapidly assess to what extent these two protein species are related, they were purified and further analyzed by peptidic mapping. Following digestion with trypsin, and the peptides further separated in two dimensions, by electrophoresis (arrow 1) followed by chromatography (arrow 2). The maps of the 37- and 35-kDa proteins are shown in A and B, respectively.

**Gene Mapping by in Situ Hybridization**—In situ hybridization was carried out on chromosomes prepared from phytohemagglutinin-stimulated human lymphocytes cultured for 72 h, the last 7 h being in the presence of 5-bromodeoxyuridine (60 µg/ml). The recombinant Bluescript II SK+ plasmid containing the 2H9 coding sequence was tritiated by nick translation (1.5 x 10⁶ dpm/µg) and hybridized to metaphase spreads (100 ng/ml), as described previously (28). The slides were coated with nuclear track emulsion (Kodak NTB₂) exposed for 18 days at 4°C, and then developed. R-banding was performed using the fluorescein-photolysis-Giemsa method.

**Immunoblotting and peptidic map of the 2H9 proteins recognized by antibody 2H9.** Left panel, a nuclear extract was resolved on an SDS-10% polyacrylamide gel which was either stained with Coomassie Blue (lane 1) or analyzed by Western blot with antibody 2H9 (lane 2). Right panel, immunoprecipitated 35- and 37-kDa proteins were separated by gel electrophoresis, radioiodinated (¹²⁵I), digested with trypsin, and the peptides further separated in two dimensions, by electrophoresis (arrow 1) followed by chromatography (arrow 2). The maps of the 37- and 35-kDa proteins are shown in A and B, respectively.

**FIG. 2. The 2H9 protein doublet is produced from two differently sized mRNAs.** Poly(A⁺) RNAs from HeLa cells were resolved on a 1.2% formaldehyde agarose gel, blotted onto nitrocellulose, and probed with the initial 330-bp cDNA fragment encoding the epitope recognized by monoclonal antibody 2H9.

**FIG. 3. In vitro translation and immunoprecipitation of protein encoded by 2H9 cDNA.** To identify the protein encoded by full-length cDNA, adequate linearized plasmids were transcribed using T3 (lanes 1; negative controls) or T7 (lanes 2) RNA polymerase, and the RNAs were translated in vitro in the presence of [³⁵S]methionine. Following analysis on an SDS-10% polyacrylamide gel, total labeled proteins (A) and proteins immunoprecipitated with monoclonal antibody 2H9 (B) were detected by fluorography; the additional bands detected in B2 are most likely due to incomplete transcription and (or) translation. 2H9 proteins immunoprecipitated from a [³⁵S]methionine-labeled nuclear extract are shown as a control (B, lane 3).

In order to check whether this 1.7-kb cDNA really encodes one or both of the 35- and 37-kDa proteins, which need a coding sequence at the most of 1000 to 1100 nucleotides. The 330-bp probe was then used to screen the oligo(dT)-primed Agt10 cDNA library. Although numerous positive plaques were obtained, only one of them contained an insert whose size (1.7 kb) was consistent with that of the 2H9 proteins and which also hybridized with both 1.5- and 2.3-kb RNAs shown in Fig. 2.

In order to check which this 1.7-kb cDNA really encodes one or both of the 35- and 37-kDa proteins, in vitro translation experiments were carried out. Fig. 3 shows that following immunoprecipitation with monoclonal antibody 2H9, a 35–37-kDa protein doublet is detected (B, lane 2), the two bands being in the same proportion as in the control nuclear extract (lane 3). These data show that the 1.7-kb cDNA apparently contains the entire coding sequence for both 35- and 37-kDa proteins. However, in terms of size our cDNA lies between the 1.5- and 2.3-kb mRNAs, thus suggesting that it is derived from the larger transcript.

**Nucleotide Sequence Analysis of the 1.7-kb cDNA**—The nu-
The nucleotide sequence of the 1.7-kb cDNA is shown in Fig. 4. This sequence contains a single long translational reading frame of 1038 bp, starting 303 bp from the 5'-terminus, all other possible frames being less than 200 bp long. The ATG codon at position 303 is flanked by nucleotides matching the translation start consensus sequence (A/G)NNATGG (29). The open reading frame encodes a 346-amino-acid protein. Two CS-RBDs (open boxes) were identified. RNP-1 and RNP-2, which are the most highly conserved motifs within the CS-RBDs, are boxed in reverse type. GGXG repeats are underlined. *, stop codon.

Analysis of the cDNA-deduced Amino Acid Sequence—The predicted 2H9 primary sequence corresponds to a protein of 346 amino acids (Fig. 4) with an expected molecular weight of 36,000, which matches the experimental values (35 and 37 kDa).
This protein sequence reveals the presence of two consensus sequence RNA-binding domains, CS-RBD I and CS-RBD II, of 80 amino acids each. Database searches (Blitz EMBO) revealed high homologies between these domains and two of the three CS-RBDs of hnRNP F (53 kDa) (31) and the closely related hnRNPH/H 9 (49 kDa) (32). The sequence alignment (Fig. 6A) reveals homologies of 89% between 2H9-RBD I and F-RBD II, 80% between 2H9-RBD I and H/H 9-RBD II, and 85% between 2H9-RBD II and F- or H/H 9-RBD II. It should be noted that protein 2H9 does not have the F- or H/H 9-RBD I counterpart; the presence of several stop codons in the 5'-untranslated region of cDNA 2H9, one of them being located nine codons upstream from the initiation codon (Fig. 4), confirms that this RBD is really absent.

The primary sequence of protein 2H9 also reveals the presence of two Gly-rich auxiliary domains (Fig. 4), one located in the central region between the two RBDs (amino acids 96–193; 32% of Gly) and the other is in the C-terminal region (amino acids 273–346; 48% of Gly), starting immediately after RBD II. Gly-rich internal and C-terminal counterparts are also present in hnRNP F (18 and 21% of Gly) and in hnRNP H/H' (27 and 23% of Gly). In contrast to the RBDs, the Gly-rich domains are less conserved (Fig. 6B); the internal domain of protein 2H9 shows 50 and 34% homology with that of hnRNP H/H' and F, respectively, whereas the C-terminal domain appears to be even less conserved, showing a conspicuous 20-amino acid insert only present in hnRNP H/H'. Further examination of the two glycine-rich auxiliary domains reveals the presence in protein 2H9 of 17 Tyr residues among which 15 are conserved in hnRNP F or H/H' either as Tyr or Phe. In the internal domain, two types of residues are well conserved too: the charged residues (Arg, Lys, Asp, and Glu) and the proline residues which are clustered within a motif showing itself a high degree of conservation (2H9 amino acids 101–117).

A comparison of the 2H9 Gly-rich domains with the C-terminal Gly-rich domain of the basic hnRNP A1, A2, and B1 proteins (33, 34) shows substantial sequence divergence, although the latter also display numerous interspersed aromatic residues, which in hnRNP A1 are essentially present within GNF/YGG/GCG consensus repeats (35). Although no such repeat is present in the 2H9, F, and H/H' proteins, one can, however, notice the presence in protein 2H9 of a short GGGX repeat (Fig. 4, underlined), which interestingly is the central motif of the RGGXGGR repeat present in the G4R domain of the Gly-rich region of human nucleolin and fibrillarin and yeast NSR1, which are all known to be involved in pre-RNA proc-
In previous studies we showed that in normal HeLa cells the 2H9 proteins are associated with hnRNP complexes (including pre-mRNP) and are off these structures following a 10-min stress at 45 °C, thus suggesting that they might be involved in a mechanism that turns splicing on and off (1). To assess this hypothesis we first tested in a direct way whether these proteins are really involved in splicing. Therefore, we checked whether specific antibodies can directly inhibit an in vitro splicing system comprising an 854-nucleotide pre-mRNA synthesized from plasmid pSp4, which contains the natural adenoviral E1A sequence (21). In the salt conditions indicated under “Experimental Procedures,” a single 114-nucleotide intron is excised from this pre-mRNA, giving rise to a 13S mRNA, the final splicing product after 5.2% polyacrylamide gels. Lane 1, initial pre-mRNA; Lanes 2 and 8, control splicing reactions without antibody; Lanes 3–7 and 9–11, splicing reaction after preincubating the nuclear extract with nonrelevant 5G4 monoclonal antibody (mAb-NR, lane 3) or polyclonal IgG (pAb-NR, lane 6), with original 2H9 monoclonal antibody (mAb-2H9, lane 4), with native (lanes 5 and 9), heat-denatured (lanes 7, lane 10), or DTT-treated (lane 11) 4A8 anti-peptide H1 monoclonal antibody (mAb-H1), and with anti-peptide H1 polyclonal antibody (pAb-H1, lane 7). The transcript (Tr), exon 1 (E1), and the 13 S mRNA (E1:E2) are indicated in the middle. A and B present two different experiments, in which two different nuclear extracts were used.

Additional data base searches for potential post-translational modifications showed that 2H9 sequence contains one possible glycosylation site on the Asn residue at position 307 and eight possible phosphorylation sites. However, we have not yet been able to show experimentally either of these modifications in HeLa cells.

**2H9 Proteins and In Vitro Splicing—** In previous studies we showed that in normal HeLa cells the 2H9 proteins are associated with hnRNP complexes (including pre-mRNP) and are off these structures following a 10-min stress at 45 °C, thus suggesting that they might be involved in a mechanism that turns splicing on and off (1). To assess this hypothesis we first tested in a direct way whether these proteins are really involved in splicing. Therefore, we checked whether specific antibodies can directly inhibit an in vitro splicing system comprising an 854-nucleotide pre-mRNA synthesized from plasmid pSp4, which contains the natural adenoviral E1A sequence (21). In the salt conditions indicated under “Experimental Procedures,” a single 114-nucleotide intron is excised from this pre-mRNA, giving rise to a 13 S mRNA, the final splicing product we will also refer to as E1:E2.

Preincubating a nuclear extract with monoclonal antibody 2H9 only led to a weak but reproducible inhibition of splicing in vitro when compared with nonrelated monoclonal antibody 5G4 (mAb-NR) from our library (Fig. 7A, compare lanes 4 and 3). Since these data relied on a single antibody with relatively low affinity, we raised both polyclonal and monoclonal antibodies against synthetic peptides representing different regions of the 2H9 primary sequence. The first antibodies we obtained were two monoclonals (4A8 and 1C9) and one rabbit polyclonal antibody, all directed against peptide H1 (amino acids 161–174) which is not conserved in hnRNP F and H/H′ (see Fig. 6B). As the original 2H9 monoclonal antibody (1), the anti-peptide antibodies only recognize the 2H9 proteins in a nuclear extract or in purified hnRNP complexes. Preincubating a nuclear extract with any of the three previously immunopurified anti-peptide antibodies severely inhibited the 13 S mRNA accumulation. Fig. 7A shows the inhibition we obtained when using anti-peptide H1 monoclonal antibody 4A8 (mAb-H1, lane 5) and anti-H1 polyclonal antibody (pAb-H1, lane 7). In comparison with control splicing, only small amounts of 13 S mRNA are detected. Moreover, exon 1 does not further accumulate, which indicates that inhibition occurs during spliceosome assembly. Using nonrelated monoclonal antibody 5G4 (mAb-NR, lane 3), nonrelated polyclonal IgGs (pAb-NR, lane 6), or mAb-H1 denatured either by heat (65 °C for 10 min; Fig. 7B, lane 10 (37, 38) or dissociated with DTT (10 mM at 30 °C for 30 min; Fig. 7B, lane 11) relieves splicing inhibition, thus showing that the inhibition we observe is due to specific antibody-antigen interactions. Additional splicing assays carried out with rabbit β-globin pre-mRNA substrate containing intron 1 led to similar splicing inhibition (not shown).

We further carried out specific depletion assays that were designed for further complementation. Experiments performed by using purified anti-peptide antibodies coupled to Sepharose-CNBr actually allowed removal of more than 90% of the 2H9 proteins from the nuclear extracts, as judged by Western blot analysis. However, such depleted extracts were still totally active in splicing in vitro (not shown) (see “Discussion”). The 2H9 Proteins Are Missing in Splicing Deficient Nuclear Extracts from Briefly Stressed Cells— Nuclear extracts from cells heat-shocked for 1–2 h at 43.5 to 45 °C were earlier shown to be inactive in splicing, which correlated with U-snRNP disruption (17). In fact, hnRNP complexes are modified as early as 10 min following a heat shock at 45 °C, since we previously found that 2H9 proteins and also a 72.5–74-kDa protein doublet, we subsequently showed to be hnRNP M proteins (22, 39), leave the hnRNP complexes and strongly bind to the nuclear matrix (1, 20). Recently we have shown that nuclear extracts prepared from cells stressed in these conditions are in fact already splicing defective, but interestingly the U-snRNPs appear to be still active (22). In order to find out what might explain this loss of splicing activity, we compared the protein content of extracts from normal and stressed cells first by SDS-PAGE analysis, which did not reveal any significant difference (see also Fig. 8A, panel 1). We then screened by Western blotting our anti-hnRNP monoclonal antibody library, which includes anti-SR splicing factor 9G8 antibody (40), and in addition we tested other antibodies like anti-hnRNPC (4F4) and anti-hnRNP U (3G6). This latter analysis only revealed two differences, one of them being that the hnRNP M proteins are clearly missing in extracts from stressed cells (22).

The second difference is that the 2H9 proteins are also missing in nuclear extracts prepared from cells heat-shocked at 45 °C for 10 min (Fig. 8A, panel 2). In fact, this was not quite unexpected since we earlier showed that, within HeLa cells, this protein doublet behaves exactly as the hnRNP M proteins with respect to heat shock (1, 20). Since extracts from such stressed cells are totally inactive in splicing in vitro (Fig. 8B, lanes 1 and 2), we then tried to see whether splicing might be restored by complementation with the purified protein. Since the 2H9 proteins could not be efficiently immunopurified from normal nuclear extracts, we expressed the recombinant protein in E. coli (Fig. 8C). Whatever the recombinant protein we used, His-tagged or not, purified from the soluble fraction or from inclusions, complementation only led to a faint but nevertheless reproducible 13 S mRNA band (Fig. 8B, lane 4). Further complementation with both 2H9 and hnRNP M recombinant...
proteins together did not improve splicing efficiency (not shown). Complementing such an inactive nuclear extract with an S100 extract, which contains 2H9 proteins but no SR factors (40), significantly restores splicing (lane 3), thus showing that the SR factors in the extract from stressed cells are still active; it should be noted that the weaker 13 S mRNA band is due to the fact that in this assay we could only use half the amount of nuclear extract present in a standard assay.

The 2H9 Proteins Are Also Detected in Nuclear Bodies—By indirect immunofluorescence we have previously shown that in normal HeLa cells the 2H9 proteins are not available for the original 2H9 monoclonal antibody, whereas cells heat-shocked for 10 min at 45 °C reveal a bright nuclear signal distributed as numerous small granules as shown in Fig. 9D (inset) (1). Later on, when routinely supplementing cell culture medium with 10% fetal calf serum instead of the newborn and fetal calf serum combination (7.5:2.5%) used previously, we still observed the previously described signal, but in both normal and stressed interphasic cells we also detect a few brightly stained nuclear bodies, which are often present as pairs, close to the nucleoli (Fig. 9, A and D). These data were obtained with monoclonal antibody 2H9, anti-peptide H1 monoclonal antibodies (1C9 and 4A8), as well as with other monoclonal antibodies we obtained more recently and which are directed against other peptides. These nuclear bodies are not nuclear speckles, known to contain several splicing factors, but their size and shape rather point to coiled bodies, which were shown to contain splicing factor U2AF (41, 42). Double immunocytofluorescence assays using an anti-peptide H1 monoclonal antibody (Fig. 9, A and D) and a rabbit polyclonal anti-coilin antibody to label coiled bodies (43, 44) (Fig. 9, B and E), however, show that the nuclear bodies revealed with the anti-H1 antibody are not coiled bodies (Fig. 9, C and F).

Following these observations, we went back to the nuclear extracts in order to check whether any of their properties might also be serum-dependent. Therefore, we grew cells in 10% fetal calf serum and prepared nuclear extracts from normal and heat-shocked cells. In fact, when assayed for in vitro splicing capacity and for the presence of 2H9 proteins, as well as for hnRNP M proteins (22), these extracts did not reveal any difference with the extracts described above and which were prepared from cells grown in 10% newborn calf serum.

Southern Blot Analysis and Chromosomal Mapping—Southern blot analysis was performed on human cellular DNA digested with either EcoRI (1 site in 2H9 cDNA) or TaqI (1 site) and further blotted on Highbond N+. Hybridization under normal stringency conditions, using as a probe a 1.7-kb cDNA labeled with 32P by random priming (23), revealed two EcoRI bands and two TaqI bands. Cellular DNAs from different individuals from the CEPH (Centre d’Etude du Polymorphisme Humain) family panel all revealed this pattern, no polymorphism being observed (not shown).

In situ hybridization was performed on metaphasic human lymphocytes as described before (28). Within the 100 cells examined, 154 silver grains were associated with chromosomes, and 32 of these (21%) were located on chromosome 10; the grain distribution was nonrandom since 24 out of 32 (75%) mapped to the q22 band of the long arm of chromosome 10. These results allow us to map the 2H9 gene to the 10q22 band of the human genome (Fig. 10). These results, along with the Southern blot data, suggest a single 2H9 locus in the human genome.

DISCUSSION

In the present work we investigate the structure and function of the 2H9 proteins (35–37 kDa) we have previously shown to be involved in minute and reversible alterations of hnRNP complexes triggered by a 10-min heat shock at 45 °C (1). The 1.7-kb cDNA we isolated encodes genuine 2H9 proteins (Fig. 3). It further hybridizes with two poly(A+)-mRNAs of 1.5 and 2.3 kb (Fig. 2), which probably differ by their 3’-untranslated sequence, due to differential use of two polyadenylation signals. Moreover, both 35- and 37-kDa protein species are produced by the single mRNA transcribed from the 1.7-kb cDNA. These data, together with those obtained by Southern blotting and chromosomal mapping, point to the 2H9 proteins as products from a single gene.

Since both 35- and 37-kDa protein species are synthesized in vitro from a single mRNA species, it is likely that one of the proteins derives from the other by post-translational modifications. Alternative use of the second but less favorable ATG codon (amino acid position 5) (29) might be another possibility, which in addition would explain the lesser proportion of the 35-kDa protein. Furthermore, the 35-kDa protein might also occur by proteolytic cleavage of the 37-kDa species, as several potential cleavage sites for trypsin-like (Arg) and chymotrypsin-like (Met, Trp, Tyr) proteases are present within the rather hydrophilic C terminus.

The predicted 2H9 primary sequence reveals that protein 2H9 is a new member of a family of related but distinct hnRNP proteins that includes hnRNP H/H’ (49 kDa) and hnRNP F (53 kDa) (31, 32). HnRNP H and H’, which are homologous at 96%, share 75% identity with the hnRNP F, including three highly
conserved RBDs. As protein 2H9 is lacking the upstream RBD and only shows 57 and 59% homology with the comparable sequences in hnRNP F and H/H', they clearly appear to be the most divergent within that family. This actually correlates with the fact that affinity chromatography of nucleoplasmic proteins on poly(rG) selected hnRNP E, F, H, and M but apparently not the 2H9 proteins (31). Indeed, although the 2H9 RBDs are highly conserved in hnRNP F and H, differences appear for instance within loop 3 (Fig. 6A) and its adjacent sequences (Fig. 6B), which might well confer a somewhat different RNA sequence specificity to the 2H9 proteins. Such structural similarities between the corresponding RBDs of hnRNP proteins 2H9, F, H, and H' are likely to provide more flexibility in modulating RNA binding, which for a growing number of hnRNPs appears to underly an RNA annealing or chaperone activity designed to bring RNA sequences in a position and a configuration within the splicing complex so as to become available for splicing reactions (45–47). Despite these structural relationships it should be noted that these proteins have different chromosomal locations in the human genome, mapping to locus 10q11.21/22 (hnRNP F), 5q35.3 (hnRNP H), Xq22 and/or 6q26 (hnRNP H') (32), and 10q22 (2H9).

Another feature protein 2H9 shares with hnRNP F and H is the presence of a Gly-rich auxiliary domain located between RBD I and II (2H9) or RBD II and III (hnRNP F and H) and a second one in the C-terminal region. Interestingly, aromatic amino acid residues (essentially Tyr) interspersed along these two domains are mostly conserved, thus suggesting a role in structure and function. Moreover, as hnRNP F and H, protein 2H9 displays a sequence similar to a 30–40-amino acid domain present in hnRNP A1 and A2 and which is known to localize hnRNP A1 to the nucleus (48, 49). Interestingly, a simplified hnRNP A1/A2 consensus, YX₂FX₂YX₃FX₅X₇–₉Y (X is often G and N), deduced from the domain defined by Weighardt et al. (49), matches very well the hnRNP F/H and 2H9 consensus we determined as YX₂Y/FX₂YX₃FX₅Y; in protein 2H9 this consensus is starting at position 147 (Fig. 6B). Numerous interspersed aromatic amino acids some of them being in GGXG repeats is also one of the features of the C-terminal Gly-rich domains of hnRNP A1, A2, and B1 proteins. By analogy with the properties of the extensively studied hnRNP A1 domain (35, 36, 46), one can assume that the 2H9 proteins might also

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**Fig. 9. Confocal microscopy.** Distribution of the 2H9 proteins and p80-coilin in normal and stressed HeLa cells. Normal cells (A–C) and cells heat-shocked for 10 min at 45°C (D–F) were processed for double immunofluorescence as described under “Experimental Procedures.” 4A8 anti-peptide H1 monoclonal antibody was used for the detection of the 2H9 proteins (A and D, red staining), and anti-coilin polyclonal antibody to detect the coiled bodies (B and E, green dots). C (left part) and F are confocal overlays of A and B and D and E, respectively. C (right part), Hoechst staining of the nucleus (N) shown in A–C (left part). The arrows point to nuclear bodies containing 2H9 proteins. Due to the strong staining of the nuclear bodies in heat-shocked cells, the confocal micrograph shown in D is underexposed compared with A. The micrograph inserted in D shows a nucleus examined with a conventional photomicroscope fitted for epifluorescence and photographed at normal film exposure (Technical Pan 2415, Eastman Kodak). The thin arrows point to a pair of nuclear bodies. The image size is a 2.5-fold reduction when compared with the bar in F.

**Fig. 10. Location of the 2H9 gene to human chromosome 10 by in situ hybridization.** All procedures were as described under “Experimental Procedures.” Diagram of chromosome 10 indicates the distribution of labeled sites.
have the capacity to bind RNA unspecifically, which is thought to allow migration along the RNA, until RBDS reach the specific high affinity RNA sequences (36). Furthermore, we observed earlier that the 2H9 proteins can bind the major or basic hnRNP proteins (1), which is in line with studies showing that Gly-rich domains promote protein-protein interactions (35, 36). hnRNP proteins (1), which is in line with studies showing that Gly-rich domains promote protein-protein interactions (35, 36). Unlike hnRNP A1, A2, and B1 which contain a single Gly-rich domain, the 2H9 proteins as well as hnRNP F and H/H’ exhibit two of them which might confer these proteins increased functional potentials linked to both protein-RNA and protein-protein contacts.

As to the function of the 2H9 proteins, we show that three anti-peptide H1 antibodies (two monoclonals and one polyclonal) did inhibit in vitro splicing of an adeno virus pre-mRNA, which points to direct or indirect involvement of these proteins in splicing in vitro. Unfortunately, splicing inhibition in vitro could not be confirmed by specific immunodepletion, although only low amounts of 2H9 proteins were left in the extracts. In fact, this result might well be explained by the extensive structural relationships between protein 2H9 and hnRNP F and H/H’, which are likely to allow some interchangeability; indeed, 2H9-depleted nuclear extracts may still contain hnRNP F and H/H’ which are not recognized by the anti-peptide H1 antibodies and might then functionally replace protein 2H9 in splicing assays in vitro. An additional clue in favor of the involvement of the 2H9 proteins in splicing is provided by our heat shock approach. Indeed, it turns out that such extracts can be viewed as resulting from an in vivo depletion process since they are splicing deficient, which interestingly correlates with the absence of hnRNP M (22) and that of the 2H9 proteins too. This correlation makes all the more sense since the absence of these two protein families is in line with their behavior in vivo, switching simultaneously from the hnRNP complexes to the nuclear matrix, briefly upon the onset of a heat shock at 45 °C (1, 20). However, attempts to restore the splicing capacity of nuclear extracts from stressed cells were hardly conclusive, either due to the fact that the recombinant 2H9 and hnRNP M proteins we used are not fully representative of the native proteins, especially with regard to conformation, post-translational modifications, and number of isoforms, or because the process is really more complex in the sense that other proteins, for instance the related hnRNP F and H/H’, are perhaps also missing in the extract. So, taken together, our investigations provide a series of convergent data supporting the idea that hnRNP proteins 2H9 (this study) and hnRNP M (22) are directly or indirectly involved in the splicing process and further participate in heat shock-induced splicing arrest by leaving the hnRNP or splicing complexes.

With this novel hnRN P protein 2H9, which is closely related to hnRNP F and H/H’, and also linked to the basic hnRNP A1, A2, and B1, we further emphasize the complex structural relationships occurring within the hnRNP protein population and which most likely reflect a great flexibility in regulating and tuning the splicing mechanisms. For instance, there are now evidences that various hnRNP proteins can influence 5’ splice site selection in alternative splicing. HnRNP A1 does so by antagonizing the SF/ASF and SC/35 splicing factors, which has been shown in vitro (46, 51) and in vivo (52, 53). Interestingly, hnRNP F participates in the inclusion of neuron-specific alternative exon N1 in mouse c-src mRNA (54). Finally, it has also been shown that Drosophila hnRNP protein hrp48 and a 97-kDa RNA binding protein referred to as P element somatic inhibitor are involved in the regulation of P element pre-mRNA splicing (55). In this context of hnRNP-protein relationships, it is also worth mentioning that two monoclonal antibodies from our anti-hnRNP library recently allowed us to identify two hnRN P-associated proteins showing a nuclear speckled distribution similar to that of splicing factors and which share structural features with the multifunctional adeno viral 72-kDa protein (56).

We finally showed that changing the cell culture conditions, in this case fetal calf serum concentration, is sufficient to partially modify the behavior of the 2H9 proteins. In fact, in addition to the previously observed nuclear distribution (1), we also detected their presence in nuclear bodies that do not colocalize with coilin and therefore are not coiled bodies according to the currently accepted criteria (43, 44). Actually, an increasing number of hnRNP-complex components are found to be also present in nuclear bodies, which comprise five different classes including the coiled bodies (57). Indeed, 2H9 proteins, along with hnRNP M (22), K (58), and L (59) proteins, are also present in yet undetermined nuclear bodies, whereas U-snRNPs and splicing factor U2AF also localize in coiled bodies (41, 42, 60). This further fits in a more recent concept of the splicing apparatus, which emphasizes the fact that some of its components are actually present in several nuclear compartments other than pre-mRNA processing sites (61–63).

Whether upon increasing fetal calf serum concentration 2H9 proteins switch to these nuclear bodies or just become accessible to the antibody is not known yet. However, this aspect of the behavior of these proteins shows that they most likely also intervene in mechanisms enabling cells to physiologically adjust to various environmental changes, which might not necessarily correspond to what is usually referred to as stress situations.

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