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hnRNPs H, H′ and F behave differently with respect to posttranslational cleavage and subcellular localization

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Abstract hnRNPs H, H′ and F belong to a subfamily of the hnRNPs sharing a high degree of sequence identity. Eukaryotic expression and specific C-terminal antibodies were used to demonstrate great variation in the intracellular fate of the proteins. hnRNPs H and H′ become posttranslational cleaved into C-terminal 35 kDa proteins (H_C, H'_C) and possibly into N-terminal 22 kDa proteins. No detectable cleavage was observed for hnRNP F. hnRNP H/H′ is almost exclusively localized to the nucleus of many cell types while hnRNP F varies from a predominant nuclear localization in some cells to a predominant cytoplasmic localization in other cells. The different fates may reflect differences in functional roles that so far only have included nuclear functions. The presence of significant quantities of hnRNP F in the cytoplasm of many cells indicates that it also may have a functional role here.

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Key words: hnRNP; Posttranslational modification; Proteolytic cleavage; Quasi-RNA recognition motif; RNA recognition motif; Eukaryotic; Prokaryotic expression

1. Introduction

The heterogeneous nuclear ribonucleoproteins (hnRNPs) constitute a family of more than 20 proteins designated with letters from A to U [1]. The proteins are characterized by having a modular structure possessing one or more RNA binding domains together with one or more auxiliary domains that may mediate protein-protein interactions. Isoforms of some of the hnRNPs derive by alternative splicing of their pre-mRNA while other hnRNPs are reported to undergo various forms of posttranslational modifications. Of the several RNA binding motifs occurring among these proteins the most common is the RNA recognition motif (RRM) [2] consisting of about 90 amino acids with two consensus sequences, an octapeptide sequence, RNP-1, and a hexapeptide sequence, RNP-2.

Recently, we and others [3–5] have identified a subfamily of the hnRNPs consisting of hnRNP H, H′, F and 2H9 characterized by a slightly different structure of the RRM domains that have tentatively been termed quasi-RRMs (qRRMs) until the three-dimensional structure has been more firmly determed [4]. The hnRNPs are mainly involved in the metabolism of pre-mRNA [1]. The specific functions of the individual members are, however, unknown in detail. So far, only nuclear functions have been reported for hnRNPs H, H′, F and 2H9 where hnRNP H and hnRNP H′ are part of the nuclear matrix proteins [6] and stimulate pre-mRNA cleavage and polyadenylation [7]. hnRNP H is a component of a splicing enhancer complex [8] and participates in alternative splicing [9]. hnRNP F participates in pre-mRNA splicing [10] and interacts with the nuclear cap binding protein complex, CBC [10,11] and hnRNP 2H9 acts in the splicing process and also participates in early heat shock-induced splicing arrest by transiently leaving the hnRNP complex [5].

For further functional studies it is important to have antibodies specific to each of the proteins. Due to the high degree of sequence identity among the members of this subfamily previous antibodies have not been able to distinguish between hnRNPs H/H′ and F [3,4]. Here we have raised antibodies against C-terminal peptides of hnRNP H/H′ and F and analyzed the intracellular fate of the proteins in cultured cells as well as in various normal and pathological tissues. We show that, in spite of the high degree of sequence similarity among the proteins, hnRNP H/H′ but not hnRNP F is subjected to posttranslational proteolytic cleavage and that the subcellular localization of hnRNP H/H′ is very different from hnRNP F in a number of tissues. The differences in fate between the proteins likely represent differences in their functions.

2. Materials and methods

2.1. Culturing of cells and labelling with L-[^{35}S]-methionine and L-[^{35}S]-cysteine

MRC-5 [12], MRC-5 V2 [13] and COS-1 [14] cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX I supplemented with 10% (vol/vol) fetal bovine serum and antibiotics (penicillin at 100 units/ml and streptomycin at 100 μg/ml) from Life Technologies. Labelling of cells in vivo with L-[^{35}S]-methionine and L-[^{35}S]-cysteine was performed in labelling medium constructed by mixing 2.5 ml of DMEM (Life Technologies), 41.5 ml DMEM without methionine, cysteine and glutamine (Sigma), 0.5 ml GlutaMAX I Supplement (Life Technologies), 5 ml dialyzed fetal bovine serum (Life Technologies) and 0.5 ml penicillin/streptomycin (Life Technologies). Labelling was performed in this medium with the addition of 0.1 mCi/ml of Pro-mix (SJQ 0079) from Amersham.

2.2. Production of antibodies against hnRNP H/H′ and F

Peptides for immunization were obtained from Neosystem (Strasbourg, France). Two peptides were synthesized: peptide SP961010, VLQENSSDFQSNIA corresponding to the 14 C-terminal amino acids, Val^{436}-Ala^{449}, in hnRNP H (anti-H/H′) and peptide SP961174, KRAAQATYSGLESQSV corresponding to 14 internal amino acids, Ala^{384}-Val^{397}, at the C-terminal of hnRNP F (anti-F) with the addition of Lys-Arg in the N-terminal. Both peptides were coupled to keyhole limpet hemocyanin and injected in female rabbits (2–2.5 kg) purchased from the State Serum Institute, Copenhagen, Denmark. Immunization was performed essentially as described [15]. The animals were bled by vein puncture and non-specific sera were purified, first on a protein A column and then on a column with

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Abbreviations: CBC, cap binding protein complex; DMEM, Dulbecco's modified Eagle's medium; hnRNP, heterogeneous nuclear ribonucleoprotein; RRM, RNA recognition motif; qRRM, quasi-RRM

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immobilized peptide. The antibody was eluted with 0.1 M glycine buffer (pH 2.7) and neutralized with 1 M Tris-HCl, pH 9. Peptides were immobilized by coupling to cyanogen bromide-activated EAH Sepharose 4B according to the manufacturer's description (Pharmacia, Sweden).

2.3. Eukaryotic and prokaryotic expression of hnRNPs

For eukaryotic expression the cDNAs encoding hnRNP H and H′ [4] were amplified by PCR with Pfu polymerase using the following primers in case of hnRNP H: CAC-CTC-GAG-CGT-CTT-AGC-CAC-GCA-GAA (3.1H(F) with a *Xho*I site) and TTC-GGA-TCC-TTA-TGC-AAT-GTT-TGA-TTG-AAA (3.1H(R) with a *Bam*HI site) and in case of hnRNP H′: CAC-GGA-TCC-GGT-ATC-GTT-AGA-GCT-ACA (3.1H′(F) with a *Bam*HI site) and TTC-AAG-CTT-AAG-CAA-GGT-TTG-ACT-GAT-A (hnRNPH′.R with a *Hin*dIII site). The PCR products were cut with the appropriate restriction enzymes, purified on low temperature gelling agarose and ligated into pcDNA3.1/Zeo(-) (Invitrogen, San Diego, CA, USA) cut with identical restriction enzymes. The constructs were grown in large scale and purified by the alkaline lysate method [16] followed by ultracentrifugation in a CsCl gradient. All constructs were sequenced in order to check for correct orientation and sequence of the inserts. COS-1 or MRC-5 V2 cells grown in 3 cm Petri dishes containing one coverslip were transfected overnight in DMEM without penicillin and streptomycin with 2 μg of the vector constructs using LipofectAMIN (Life Technologies). Next day the medium was exchanged with labelling medium. After 48 h the cells were rinsed with PBS (2.7 mM KCl, 1.8 mM KH_2PO_4, 10.1 mM Na_2HPO_4, 140 mM NaCl, pH 7.3) and harvested with a police rubberman. Proteins were extracted into phenol and precipitated with ethanol. The precipitated proteins were pelleted by centrifugation, vacuum dried and finally dissolved in lysis buffer for subsequent analysis by two-dimensional gel electrophoresis.

For prokaryotic expression the cDNA encoding hnRNP F was ligated into the pT7-PL vector by PCR amplification essentially as described [4]. Transformation of *Escherichia coli* cells, expression and purification of recombinant hnRNP F protein was performed essentially as described [17].

2.4. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed in the first dimension by using Immobiline DryStrip immobilized pH gradients, 3–10 non-linear (Pharmacia, Uppsala, Sweden) and in the second dimension SDS polyacrylamide gel electrophoresis. To each sample was added rehydration solution (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10 NL, 0.3% DTT, Bromphenol Blue) to a final volume of approximately 400 μl. Each rehydration solution with sample was pipetted into a slot of the reswelling tray (Pharmacia) together with an Immobiline DryStrip gel and left overnight. Electrophoresis in the first dimension was run on a Multiphor II flat bed unit essentially according to the manufacturer (Pharmacia). For the second dimension home-made horizontal polyacrylamide gels were used. After electrophoresis the gels were dried and exposed with Kodak X-ray films.

2.5. Immunoblotting

Gels were placed for 10 min in 25 mM Tris-HCl, 190 mM glycine, and 20% methanol, and proteins transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes. For immunodetection of the antigen the nitrocellulose sheets were incubated for at least 2 h at room temperature in PBS buffer (2.7 mM KCl, 1.8 mM KH_2PO_4, 10.1 mM Na_2HPO_4, 140 mM NaCl, pH 7.0) containing 0.05% Tween-20 and 5% skim milk. The membranes were washed shortly in PBS with 0.05% Tween-20. The first antibody was added and incubated for a 1 h period. After three washes in PBS with Tween-20, the blots were incubated for another hour with peroxidase-conjugated swine anti-rabbit IgG from DAKO (dilution 1:5000). Finally, the membranes were washed three times (3×10 min) in PBS with Tween-20 and developed by the enhanced chemiluminescence (ECL) technique (Amersham).

2.6. Immunohistochemistry

Slides containing a variety of normal and pathological human tissue sections, formalin fixed and paraffin embedded were deparaffinized for 1 h at 60–65°C and rehydrated in consecutive baths containing xylen (3×5 min) and graded ethanol, 99% (3×5 min), 96% (2×5 min) and 70% (1×5 min) and finally for 5 min in PBS (80.9 mM Na_2HPO_4, 21.5 mM NaH_2PO_4, 0.1 M NaCl, pH 7.5). Then they were washed in PBS with 50 mM NH_4Cl (3×10 min) and for at least 5 min in baths containing graded ethanol; 30%, 50%, 75%, 90%, 100%, 90%, 75%, 50%, 30%, before washing in PBS with 1% BSA, 0.05% saponin and 0.2% gelatin (3×10 min). The slides were incubated overnight at +4°C with the first antibody diluted in PBS containing 1% BSA. Next day after 1 h at room temperature the slides were washed in PBS containing 0.1% BSA, 0.05% saponin and 0.2% gelatin (3×10 min). Endogenous peroxidase activity was blocked by incubation for 10 min in PBS/methanol/(30% H_2O_2) (8/1/1: vol/vol/vol) before washing in PBS with 0.1% BSA, 0.05% saponin and 0.2% gelatin (3×10 min). Slides were then incubated with undiluted EnVision solution from DAKO (Glostrup, Denmark) for 1 h at room temperature and washed in PBS with 0.1% BSA, 0.05% saponin and 0.2% gelatin (3×10 min). Positive reaction was developed for 10 min with DAB chromogen tablets according to the manufacturer's description (DAKO). The slides were rinsed and washed in 50 mM Tris-HCl, pH 7.6 (2×10 min) then rinsed in water and counterstained in Mayer's hematoxylin for 10 min. Rinsing was performed in tap water for 10 min. Finally they were put for 2 min in various baths with graded ethanol; 70%, 90%, 96%, 99%, 99% and finally xylen (2×2 min). Mounting was performed with Eukitt. The specificity of the antibodies was checked by monitoring a significant decrease in staining signal with anti-H/H′ preincubated with the peptide used for immunization (SP961010) and with anti-F preincubated with recombinant hnRNP F. Non-reactivity of second antibody was checked by replacing the primary antibody with buffer.

3. Results and discussion

3.1. Specific antibodies against the C-terminals of hnRNP H/H′ and hnRNP F

The hnRNPs H, H′ and F constitute a recently identified subfamily of the hnRNPs possessing unique sequence characteristics [3,4]. The sequence similarity between hnRNPs H and H′ is very high amounting to 96% whereas hnRNPs H and F and hnRNPs H′ and F show similarities of 78% and 75%, respectively (Fig. 1). The hnRNPs are involved in the metabolism of pre-mRNA and some of them shuttle between the nucleus and the cytoplasm. So far only nuclear functions have been reported for the hnRNP H, H′ and F proteins, however, the more specific functions of this novel subfamily have not been investigated in detail. Due to the high degree of sequence similarity previous reported antibodies against these proteins show a high degree of cross-reactivity [3,4]. In order to be able to study the function of each of these proteins in further detail we set forward the production of specific antibodies against internal peptides in regions where they show a relatively high degree of sequence diversity. However, first, hnRNP H and hnRNP H′ are so closely related that they can hardly be distinguished immunologically. We therefore concentrated our effort on the production of two antibodies, one that specifically recognizes hnRNP H/H′ and another that specifically recognizes hnRNP F.

Sequence comparison reveals that the C-terminal 14 amino acids of hnRNP H are 86% identical to the corresponding residues in hnRNP H′ and that this peptide is not contained in hnRNP F (Fig. 1). We therefore used this 14 amino acid C-terminal peptide of hnRNP H for the production of a polyclonal antibody against hnRNP H/H′ (anti-H/H′). In order to make an antibody against hnRNP F we selected a region with rather low similarity to hnRNP H/H′. The overall identity is above 75% but at positions Ala^{384}-Val^{397} a 14 amino acid peptide shows only 38% identity (Fig. 1) and was thus used to make a polyclonal antibody against hnRNP F (anti-F). The antibodies were used for two-dimensional Western blotting.

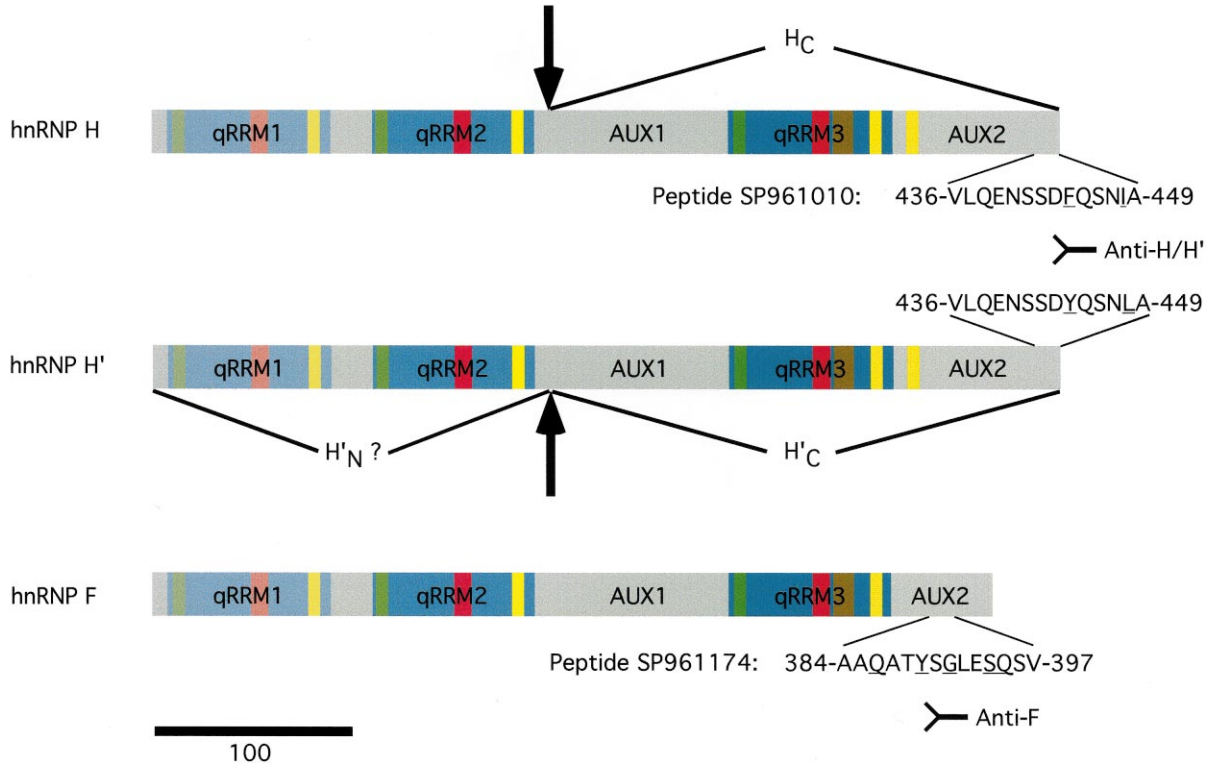

Fig. 1. Structure of the hnRNP H, hnRNP H′ and hnRNP F proteins. Each protein contains three qRRM domains (blue boxes) together with two auxiliary domains (AUX1 and AUX2, light grey boxes). The positions of the RNP-1 (red), RNP-2 (green), CS-1 (yellow) and CSR-3 (brown) consensus sequences are indicated [3,4,7]. Arrows indicate approximate positions of posttranslational cleavage sites in hnRNPs H and H′ around residue 190–200 giving the H_C and H'_C proteins and possibly the H'_N protein. hnRNP F is not cleaved. The peptides used for antibody production are indicated. The anti-H/H′ antibody was raised using the SP961010 peptide: 436-VLQENSSDFQSNIA-449 in hnRNP H. The peptide is 86% identical to the corresponding peptide in hnRNP H′ (different amino acids are underlined). The peptide is not present in hnRNP F. The anti-F antibody was raised using the SP961174 peptide: 384-AAQATYSGLESQSV-397. Underlined amino acids indicate differences to hnRNP H/H′. hnRNP H and hnRNP H′ are very similar and cannot be distinguished by the anti-H/H′ antibody.

However, for proper interpretation of these we first analyzed cells transfected with hnRNP H and H′.

3.2. Eukaryotic expression of hnRNP H′ and hnRNP H

Fig. 2A shows the two-dimensional gel of L-[^{35}S]-methionine and L-[^{35}S]-cysteine labelled control MRC-5 V2 cells transfected with the pcDNA3.1 vector devoid of insert and Fig. 2B shows MRC-5 V2 cells transfected with pcDNA3.1 containing hnRNP H′. As expected and previously shown the up-regulated hnRNP H′ protein migrates in a slightly more basic position than hnRNP H [6]. Furthermore, careful inspection of the gels from the transfected MRC-5 V2 cells revealed a very slight up-regulation of an approximately 35 kDa protein, termed H'_C (Fig. 2B) since it reacts with the C-terminal anti-H/H′ antibody (see Fig. 3). It is thus likely that H'_C represents approximately 35 kDa of the C-terminal of hnRNP H′ (Fig. 1). The up-regulation of H'_C in MRC-5 V2 cells, however, was very faint and only apparent after analysis of COS-1 cells transfected with hnRNP H′ since these cells showed a strong up-regulation of the 35 kDa H'_C protein as seen by comparing Fig. 2C with Fig. 2C. In addition, the COS-1 cells revealed a strong up-regulation of an approximately 22 kDa protein which we putatively have termed H'_N presuming that it corresponds to the N-terminal of hnRNP H′ (Fig. 1C). Finally, COS-1 cells transfected with hnRNP H demonstrated a slight up-regulation of a 35 kDa protein, termed H_C (Fig. 2E and F) since it reacts with the C-terminal anti-H/H′ antibody (see Fig. 3). However, in cells transfected with hnRNP H we could not detect any strongly up-regulated 22 kDa protein analogue to H'_N, but cannot exclude the existence of such a protein either.

The up-regulation of the H'_C, H_C and H'_N proteins might be explained by trans-activation of their genes executed by the overexpressed proteins, hnRNP H and hnRNP H′, as has recently been observed with the hnRNP K protein that functions as a transcription factor [18,19]. However, the Western blots described below show that at least H'_C and H_C are formed by proteolytic cleavage of the hnRNP H′ and hnRNP H proteins.

3.3. Two-dimensional Western blotting with anti-H/H′ and anti-F antibodies

Western blots of MRC-5 V2 cells reacted with the two C-terminal antibodies are shown in Fig. 3. First of all the anti-H/H′ antibody reacts with the hnRNP H and hnRNP H′ proteins which migrate very closely around 55 kDa with hnRNP H slightly more acidic than hnRNP H′ [6]. Two very weak spots migrating with slightly lower molecular mass also react with the anti-H/H′ antibody as indicated with arrowheads in Fig. 3B and in the inset in the upper left corner. All the protein spots shown contain the C-terminal epitope of hnRNP H/H′ (Fig. 1) and are thus likely to represent various chemical modifications of the proteins. The anti-H/H′ antibody also reacted strongly with the H_C protein spot

localized around 35 kDa as seen in Fig. 3B and as shown by a separate experiment in Fig. 3D and E. Since the antibody was raised against a C-terminal peptide of hnRNP H (Fig. 1) we find that the major spot may correspond to the C-terminal 35 kDa of hnRNP H which we consequently have termed H_C (Fig. 3E). These results thus confirmed the above described transfection studies that H'_C and H_C represent C-terminal peptides obtained by proteolytic cleavage of hnRNP H′ and hnRNP H, respectively. In addition to the reaction with the H_C protein (Fig. 3E) we also found reaction with two spots migrating with slightly lower molecular mass (arrowheads in Fig. 3E) in analogy with the reaction with the uncleaved hnRNP H and hnRNP H′ proteins (stippled box, upper left corner, Fig. 3B). These lower molecular mass proteins are analogously likely to represent low levels of modified versions of the C-terminal of hnRNP H. This may also apply to the very low levels of the protein indicated with a question mark (Fig. 2D and E).

The anti-F antibody specifically reacts with the hnRNP F protein as seen by the blot in Fig. 2C. Thus there is no indication of the presence of proteolytic cleavage products of hnRNP F.

A fact that strengthens the cleavage hypothesis is that the approximate sum of molecular masses of H'_C (35 kDa) and H'_N (22 kDa) is close to the total molecular mass of hnRNP H′ (55 kDa). Secondly, from the deduced sequence of the hnRNP H′ protein it can be calculated that if it is cleaved around amino acid number 190–200 the expected molecular masses and p*I*s of the obtained products are in agreement with the observed positions of the proteins in the two-dimensional gels, i.e. for H'_C we calculated a molecular mass of about 26–28 kDa and a p*I* of about 5.1–5.7 and for H'_N a deduced molecular mass of about 22–23 kDa and a p*I* of about 6.1–7.3. The two-dimensional gels thus confirm that H'_C has a higher molecular mass than H'_N and migrates in a more acidic position than H'_N (Fig. 2).

3.4. Structure of the cleavage products

The up-regulation of the H_C, H'_C and possibly the H'_N protein in the transfected cells may thus be explained by proteolytic cleavage of the overexpressed hnRNP H and hnRNP H′ proteins. Overexpression is not necessary for cleavage since the H_C and H'_C proteins could be demonstrated by Western blotting of both normal and transformed MRC-5 fibroblasts (not shown). It could be argued that the functional significance of cleaving the hnRNP H/H′ proteins would be to inactivate them. However, cleavage of hnRNP H′ around amino acid number 190–200 gives the H'_C protein two auxiliary domains together with the qRRM3 domain and the putative H'_N protein two qRRM domains (see Fig. 1). The structure of

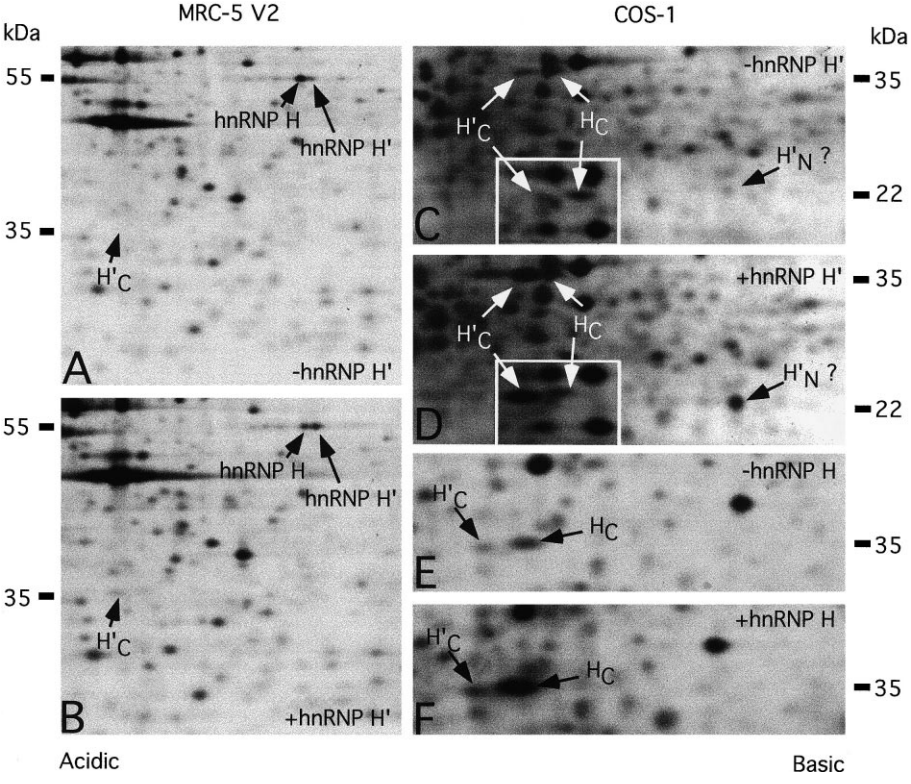

Fig. 2. Two-dimensional gels of labelled and transfected MRC-5 V2 fibroblasts (A, B) and COS-1 cells (C–F). Cells transfected with the pcDNA3.1 vector containing insert is indicated with (+hnRNP H or +hnRNP H′) while control cells transfected with the pcDNA3.1 vector without insert is indicated with (−hnRNP H or −hnRNP H′). As expected the position of the hnRNP H′ is slightly basic to the hnRNP H protein [6] seen by comparing the hnRNP H′ transfected cells (B) with the controls (A). Furthermore, a slight up-regulation was also seen of an approximately 35 kDa protein putatively assigned H'_C since it is recognized by the C-terminal anti-H/H′ antibody (see Fig. 3). Up-regulation of the H'_C protein, however, is much more apparent in hnRNP H′ transfected COS-1 cells (C, D). Insets in C and D show results from a separate experiment. Another approximately 22 kDa protein putatively assigned H'_N was also found to be markedly up-regulated in COS-1 cells transfected with hnRNP H′. This protein presumably corresponds to the N-terminal of hnRNP H′. Finally, in COS-1 cells transfected with hnRNP H a slight up-regulation of the approximately 35 kDa H_C protein was seen (E, F). H_C is recognized by the C-terminal anti-H/H′ antibody (see Fig. 3). The acidic and basic side of the isoelectric focusing is indicated as well as the approximate molecular masses.

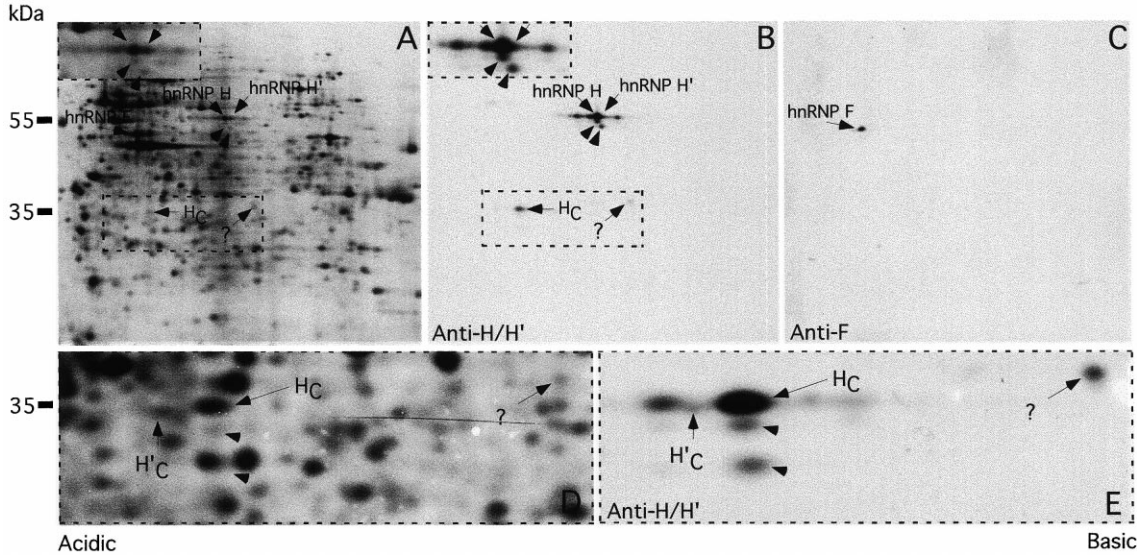

Fig. 3. Two-dimensional Western blots of MRC-5 V2 fibroblasts. The labelled blot is shown in A. B shows the Western blots reacted with anti-H/H′ and C shows the blot reacted with anti-F. The positions of hnRNPs H, H′ and F are indicated with arrows. The stippled box in upper left corner (A, B) is a magnification of the hnRNP H and hnRNP H′ region. Arrowheads show two reacting proteins migrating with slightly lower molecular mass. Central stippled box shows the 35 kDa H_C protein that reacts with the C-terminal anti-H/H′ (as seen in B). This central area is shown magnified from a different experiment in D (labelled) and in E (anti-H/H′ blot). In the magnification it is seen that two faint proteins (arrowheads), likely to represent modifications of H_C, with lower molecular masses react with the C-terminal anti-H/H′ antibody. The question mark indicates a faint reaction with an unknown protein also likely to represent a modification of H_C. C shows the Western blot reacted with anti-F. Only the hnRNP F protein reacts with the C-terminal anti-F antibody with no evidence of the presence of cleavage products. The acidic and basic side of the isoelectric focusing is indicated as well as the approximate molecular masses.

the H'_N protein would thus resemble the UP 1 protein that is obtained by proteolytic cleavage of another heterogeneous nuclear ribonucleoprotein, hnRNP A1 [20]. The UP 1 protein consists of the 195 N-terminal residues of hnRNP A1 containing two RRM domains and besides possessing a nucleic acid helix-destabilizing effect [20,21] it has also been reported to stimulate the activity of DNA polymerase α [20,22] an activity which is not contained in the hnRNP A1 protein itself [20]. It is thus likely that some of the cleavage products do have functions of their own. Further studies, however, are required in order to reveal the physiological role of these posttranslational modifications as well as those previously described such as phosphorylations [4] and methylations [23]. Finally, it should be stressed that we have positive evidence for the relatedness between H'_C and hnRNP H′ and between H_C and hnRNP H but not between H'_N and hnRNP H′. We cannot exclude the possibility that H'_N actually represents a protein trans-activated by H'_C or hnRNP H′. The level present of this protein as judged by silverstaining of the gels, however, is so low that a separate identification using, e.g. mass spectrometry becomes very difficult. Also, we unfortunately failed to produce a successful antibody against the N-terminal (Lys^{35}-Arg^{44}) of hnRNP H.

3.5. Subcellular localization of hnRNP H/H′ and hnRNP F in various tissues

We further used the specific antibodies to reveal the subcellular localization of each of the hnRNPs by immunohistochemical analyses of a variety of normal and tumor tissues. In all tissues examined we observed that the main subcellular localization of hnRNP H/H′ always was in the nucleus. Fig. 4 shows examples of this in the stratified squamous epithelium of the uterine cervix (Fig. 4A and E), in the pseudostratified columnar epithelium of the endometrium (Fig. 4G), in the islets of Langerhans (Fig. 4I), in smooth muscle cells (Fig. 4K) as well as in nerve cells (Fig. 4M). The hnRNP F protein on the contrary localizes entirely different. In the stratified squamous epithelium of the uterine cervix (Fig. 4C) the nuclei of cells in the basal layers stain strongly whereas the cytoplasm apparently contains only very low amounts (black arrows, Fig. 4C). As the cells move towards the superficial layers increasing amounts apparently accumulate in the cyto-

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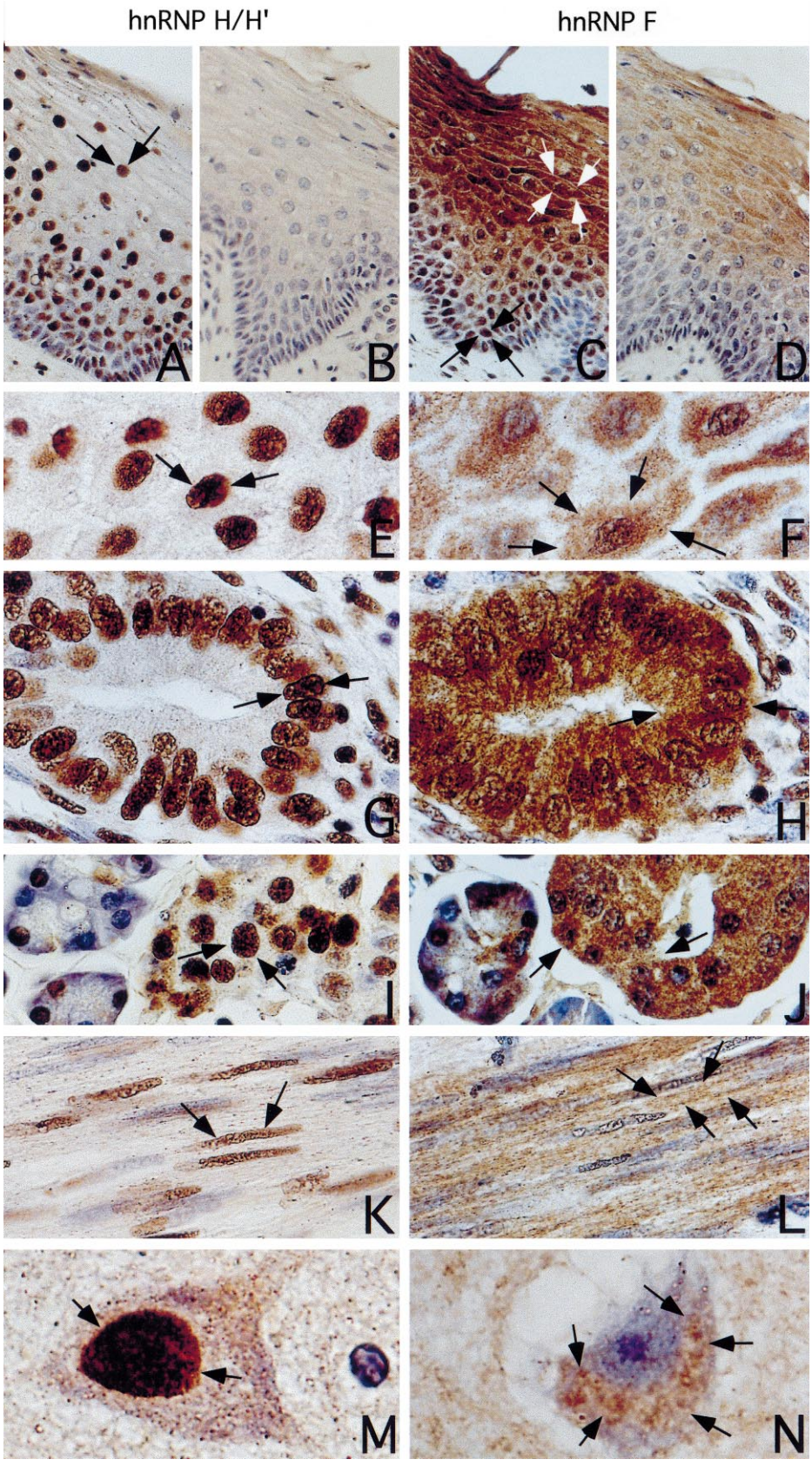
Fig. 4. Immunoperoxidase staining of various normal and pathological tissues. Stratified squamous epithelium (planocellular carcinoma) of the uterine cervix (A–F), pseudostratified columnar epithelium (adenocarcinoma) of the endometrium (G, H), islets of Langerhans (I, J), smooth muscle cells (K, L) and nerve cells (M, N). hnRNP H/H′ is almost exclusively localized to the nuclei as exemplified with the arrows in A, E, G, I, K, M. B is a control to A where the anti-H/H′ antibody prior to analysis has been preincubated with the peptide (SP961010) used for immunization. hnRNP F shows a quite different localization in the same tissues. In the stratified squamous epithelium hnRNP F localizes to the nuclei of the cells in the basal layers of the epithelium (black arrows, C) while increasingly more hnRNP F protein localize to the cytoplasm as the cells move toward the superficial layers as illustrated by the white arrows (C). D is a control to C where the antibody prior to analysis has been preincubated with recombinant hnRNP F. F shows a close view of the cells in the epithelial layer of C showing a rather even distribution of hnRNP F between the nucleus and the cytoplasm. In the pseudostratified columnar epithelium (adenocarcinoma) of the endometrium (H) as well as in the islets of Langerhans (J) an even distribution is observed with hnRNP F localized in the cytoplasm as well as in the nucleus. In smooth muscle cells (L) and in nerve cells (N) hnRNP F is apparently localized predominantly in the cytoplasm with modest amounts present in the nucleus.


hnRNP H/H'
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N

plasm so that a more even distribution is seen between the nucleus and the cytoplasm (white arrows, Fig. 4C and F). An even distribution between the nucleus and the cytoplasm is also apparent in the pseudostratified columnar epithelium of the endometrium (Fig. 4H) as well as in the islets of Langerhans (Fig. 4J). In smooth muscle cells (Fig. 4L) and in nerve cells (Fig. 4N) hnRNP F apparently distributes so that the main part is localized in the cytoplasm with more modest amounts present in the nucleus. The present results largely confirm and extend those reported by Matunis et al. [3] who used a monoclonal antibody that recognizes hnRNPs F and H (and presumably H′) and with immunofluorescence observed a localization mainly distributed in the nucleus but also to a certain extend in the cytoplasm of HeLa cells. In the light of our results it seems that the cytoplasmic staining is largely due to the hnRNP F antigen and the nuclear staining may be the result of the presence of both hnRNP F and hnRNP H/H′. Using a laser scanning confocal microscope they found the antigen to be concentrated in discrete regions in addition to having a diffuse nucleoplasmic localization of resemblance, although different, to the 'speckled' staining pattern observed with an antibody to snRNP [3].

In summary, the large majority of the hnRNP H/H′ antigens are thus localized in the nucleus, indicating that the main functions of these two hnRNPs are within the nucleus. This is in line with the observation that both are common nuclear matrix proteins [6] and possess several functions that are performed in the nucleus, including participation in splicing [8,9] and pre-mRNA cleavage and polyadenylation [7]. The hnRNP F antigen, on the other hand, besides being present in the nucleus is also found in significant quantities in the cytoplasm in a number of tissues. It is thus likely that besides the previously identified nuclear functions of hnRNP F, i.e. participation in pre-mRNA splicing [10,11] and interaction with the nuclear cap binding protein complex, CBC [11] it may also possess cytoplasmic functions due to the significant quantities of the protein localized here (Fig. 4C, F, H, J, L and N).

3.6. Conclusion

In conclusion, we have identified a number of differences in the fate of the very closely sequence related hnRNPs H, H′ and F. hnRNP H and hnRNP H′ are subjected to posttranslational cleavage where the resulting proteins may have functions on their own. Furthermore, the differences in the subcellular distribution of the proteins with hnRNP H/H′ more strictly localized in the nucleus while hnRNP F shows a localization that varies from a predominant nuclear presence to a predominant cytoplasmic presence dependent on the specific cell type may reflect differences in their functions. The functional significance of these differences now need to be investigated in further detail.

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