

The 72/74-kDa polypeptides of the 70–110 S large heterogeneous nuclear ribonucleoprotein complex (LH-nRNP) represent a discrete subset of the hnRNP M protein family

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Pre-mRNA processing in eukaryotes is thought to take place on a multitude of nuclear ribonucleoprotein (RNP) complexes, the most abundant of them being the heterogeneous nuclear (hn) RNP complexes. The identification in mammalian nuclear extracts of a novel, less-abundant 70–110 S heterogeneous RNP, named large heterogeneous nuclear RNP (LH-nRNP), has previously been reported by Aidinis, Sekeris and Guialis (1995) *Nucleic Acids Res.* **23**, 2742–2753. The structural composition of the LH-nRNP complex has been determined following the production of polyclonal antibodies against the major protein constituents of the complex, the pair of the 72/74-kDa polypeptides. In the present study evidence is shown to prove that the 72/74-kDa proteins are members of the hnRNP M protein family, hereafter referred to as 72/74(M) polypeptides. The extensive application of two-dimensional gel electrophoresis, combined with specific immunoprecipitation and immuno-

blotting assays, has allowed the assignment of the 72/74(M) proteins to a subset of the hnRNP M family, characteristic of the presence of the LH-nRNP complex and distinct from the hnRNP-associated M1–M4 components. Moreover, the immunoselection of the LH-nRNP complex from [³²P]orthophosphate-labelled HeLa cells, with the parallel application of UV irradiation, has permitted the identification of the 72/74(M) polypeptides as the sole protein constituents of the complex in direct contact with the RNA. It is proposed that LH-nRNP constitutes a discrete subset of hnRNP complexes, having a possible role in establishing specific interactions between hnRNP and nuclear-matrix protein components.

Key words: hnRNP complex, hnRNP M polypeptide, hnRNP protein, RNA-binding protein.

INTRODUCTION

The nuclear fate of precursor mRNA [pre-mRNA or heterogeneous nuclear RNA (hnRNA)] in eukaryotes involves a full range of specialized functions that refer to pre-mRNA processing (capping, methylation, polyadenylation and splicing) and to the transport of the mature mRNA to the cytoplasm through nuclear pores (reviewed in [1–3]). The importance of these functions in cell physiology is underlined by the finding of a large number of human genetic defects caused by inaccurate mRNA metabolism [4]. The major outcome of the large number of related studies is the realization that a great deal of regulation of gene expression in eukaryotes is operated at the post-transcriptional level.

Pre-mRNA maturation requires the assembly of hnRNA molecules into dynamic, multi-component structures, the most abundant of which are referred to as heterogeneous nuclear ribonucleoproteins (hnRNPs). So far, a large number of proteins (over 30) have been identified in stable association with hnRNP complexes. They comprise a group of RNA-binding polypeptides, collectively referred to as hnRNP proteins, ranging in size from 34 to 120 kDa and designated with the letters A to U [5]. hnRNP polypeptides are classified into several families and subfamilies based on shared structural/functional properties. The most abundant and best-analysed hnRNP polypeptides are those belonging to the A/B-type family, with representative species being the A1, A2/B1 and B2 proteins. This is a group of basic polypeptides, with a large number of isoforms resulting from

alternative splicing as well as from extensive post-translational modifications such as phosphorylation and methylation of arginine. They have a common modular structure with a highly conserved N-terminal RNA-binding domain (RBD) and a C-terminal auxiliary domain rich in glycine, mainly involved in protein–protein interactions [5,6]. Among the abundant hnRNP polypeptides are also species belonging to the hnRNP M [7] and L [8] protein families.

The general property of hnRNP polypeptides to bind RNA has been attributed to the presence of specific RNA-binding motifs. Three such motifs, the so-called RBD (also referred to as the RNA-recognition motif, RRM), the KH motif and the RGG box have been identified within several hnRNP proteins [9]. The RBD domain is the most commonly found and best-analysed structure, detected in either one or multiple copies within several hnRNP protein species. For example, two such motifs are found within the A/B-type and three within the hnRNP M protein families. Overall, the hnRNP proteins are found to participate in RNA–protein interactions, having a general as well as a specialized function in RNA recognition. Specific members of this large family of proteins are found to have a role in basic, as well as in alternative splicing [10,11]. Evidence is currently accumulating showing their partitioning in nucleo-cytoplasmic transport [12] and in interactions with nuclear-matrix-associated components [13,14]. Finally, the important recent findings that an increasing list of hnRNP polypeptides has a multiplicity of functions, including (in addition to RNA processing) DNA replication,

Abbreviations used: hnRNA, heterogeneous nuclear RNA; hnRNP, heterogeneous nuclear ribonucleoprotein; snRNP, small nuclear RNP; LH-nRNP, large heterogeneous nuclear RNP; 2-D, two-dimensional; NEPHGE, non-equilibrium pH-gradient electrophoresis; RBD, RNA-binding domain.

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transcription and translation, have implicated their role in the coupling and co-ordination of different metabolic pathways (reviewed in [15]). Thus hnRNP proteins are currently considered important gene regulators implicated in almost every aspect of mRNA biogenesis. From the above, the importance of pursuing research on protein species belonging to the hnRNP family is justified, as many aspects of their biological mode of action remain largely unexplored.

Apart from hnRNP complexes, the other well-scrutinized nuclear RNA/protein assembly is the much less abundant spliceosomal complex. This is also a multi-component, dynamic structure that is active in RNA splicing and is composed of the spliceosomal small nuclear RNP (snRNP) complexes (U1, U2, U4/U6 and U5) in association with specific members of the hnRNP proteins, as well as with additional splicing factors [1]. It is the interplay of the many different components of hnRNP and spliceosomal complexes that is thought to be responsible for the selective and accurate maturation of the plethora of pre-mRNA molecules in eukaryotes. How exactly this extremely complicated task is accomplished is, to a large extent, still a matter of speculation. Recent studies have pointed out the existence of several forms of hnRNP complexes with different sets of hnRNP proteins that could perform specialized functions in RNA maturation [14,16]. The identification of new subsets of RNP complexes with distinct protein and RNA composition will thus strengthen the notion of the existence of a multitude of functionally discrete protein/RNA assemblies in the cell nucleus. Along these lines, we have previously reported the identification, within fractionated nuclear extracts of rat and human origin, of a novel RNP complex of 70–110 S that we referred to as large heterogeneous nuclear RNP (LH-nRNP) [17]. A major protein constituent of the LH-nRNP complex was the 72/74-kDa protein doublet. The use of polyclonal antibodies, produced in guinea pigs, with the ability to react with the pair of 72/74-kDa polypeptides, has permitted the set-up of specific immunoprecipitation assays and the identification of its associated protein and RNA components. The LH-nRNP complex has, thus, been found to have a simple protein pattern, compared with the hnRNP and snRNP assemblies, consisting, in addition to the major 72/74-kDa protein species, of three stable associated polypeptides with apparent molecular sizes of 110, 61 and 59 kDa. The RNA constituent of the LH-nRNP complex was found to correspond to a subset of the hnRNA contained within immunoselected hnRNP complexes, exhibiting a greater overall stability and larger size. Application of the antibodies against the 72/74-kDa proteins in an *in vitro* RNA-splicing reaction has provided initial evidence for their functioning in RNA splicing [17]. In the present study, we present additional findings that allow us to conclude that the 72/74-kDa polypeptides of the LH-nRNP complex are members of the hnRNP M protein family and that they define a discrete subset of isoforms belonging to this particular hnRNP protein family.

MATERIALS AND METHODS

Nuclear-extract preparation

Purified nuclei, of rat liver origin, were extracted in a buffer containing 10 mM Tris/HCl, pH 8.0, 140 mM NaCl and 1 mM MgCl₂, according to the protocol given in [17]. Fractionation of the nuclear extract was on the combined sucrose/glycerol gradients also described previously [17]. Material contained in the 40 S and 70–110 S sucrose-gradient fractions was obtained by sedimentation at 70 000 g for 18 h at 4 °C.

Monolayer cultures of HeLa cells, grown in minimal essential medium with 10 % fetal calf serum, were used to obtain radio-

labelled nuclear extracts. Cell labelling, with either [³⁵S]Met or [³²P]orthophosphate, was exactly as described before [17]. Preparation of nuclear extracts was according to the protocol given in [18].

UV cross-linking

Monolayer cultures of HeLa cells that had been labelled with [³²P]orthophosphate for 17 h were placed in PBS buffer and exposed to UV irradiation, at 5 cm distance from a 15 W germicidal lamp (Philips TUV), for 1 min at room temperature. Cells were then scraped from the dishes, collected in cold PBS and pelleted at 2000 g for 10 min at 4 °C. Preparation of nuclear extracts was as in [18].

Antibodies

The production, in guinea pigs, of the α -72/74I and α -72/74J sera has been described before [17]. For the new antibody population (α -72/74B serum), the proteins contained in the 70–110 S sucrose/glycerol-gradient fractions were first subjected to two-dimensional (2-D) gel analysis (see below). Following identification of the 72/74-kDa polypeptides on the briefly stained Coomassie gel, the spot corresponding to the 74-kDa protein species was then excised. The gel slice was dehydrated and pulverized and used to immunize guinea pigs, applying the same protocol as for the production of the first antibodies.

The monoclonal 1D8 antibody made against the human recombinant hnRNP M4 protein and able to react with all known M variants (M1–M4) [7], was the gift of M. Swanson (University of Florida, Gainesville, FL, U.S.A.). The monoclonal antibodies 4F4 and 4D11 with specificity for the C1/C2 and L hnRNP proteins, respectively [8,19], were provided by Professor G. Dreyfuss (University of Philadelphia, Philadelphia, PA, U.S.A.).

Gel electrophoresis and immunoblotting

For protein analysis, the 2-D non-equilibrium pH-gradient electrophoresis (NEPHGE)-SDS/PAGE system of O'Farrell et al. [20] was applied throughout this study. An ampholine range of pH 3–10 (Pharmacia) was used in the first dimension and resolution of proteins by SDS/PAGE in the second dimension was on either 8 % or 10 % polyacrylamide gels (for the rat and HeLa proteins, respectively). The gels were either stained with Coomassie Brilliant Blue to directly visualize the proteins or subjected to electrotransfer and subsequent immunoblotting according to protocols described previously [17]. The guinea pig sera were used at 1:100 dilution with the horseradish peroxidase colorimetric immunodetection system and at 1:1000 dilution with the enhanced chemiluminescence (ECL) reaction system (Amersham). The monoclonal antibodies were applied, in the ECL system, at 1:1000 dilution.

Immunoprecipitation assays

All immunoprecipitation reactions were performed in NET-2 buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.5, and 0.05 % Nonidet P40) at 4 °C, as before [17]. For protein analysis on the 2-D gel system, the immune pellet was dissolved directly in the lysis buffer specified by the NEPHGE-SDS/PAGE system [20].

In the experiment where the immunoprecipitation reactions were performed on nuclear extracts from [³²P]orthophosphate-labelled HeLa cells, the immune pellets were first treated with protease-free RNase A (30 μ g per assay) to digest the bulk of the RNA, according to the protocol given in [21]. Following phenol

extraction, the proteins were then obtained by acetone precipitation.

Peptide sequence analysis

The steps involved in the partial amino acid sequence analysis of the rat 74-kDa polypeptide are outlined in the Results section. Sequencing was performed at the Max-Delbrück Zentrum für Molekulare Medizin, Berlin, Germany.

Data presentation

The gel, blots, fluorograms and autoradiograms shown in Figures 1, 3, 4, 5 and 6 were scanned using an Epson GT-5500. The software used was Microsoft Photo Editor for Windows 98.

RESULTS

Antigenicity of the 72/74-kDa polypeptides of the LH-nRNP complex

To identify the nature of the 72/74-kDa polypeptides, marker-protein constituents of the LH-nRNP complex, we relied on the extensive application of specific immunochemical assays. The use of antibodies with a well-defined specificity for the 72/74-kDa antigenic protein species was, therefore, desirable. In our initial studies we had employed two guinea pig sera (72/74I and 72/74J) with antibodies against the 72/74-kDa polypeptides [17]. The immunogenic material was the SDS-gel-excised 72/74-kDa protein doublet of the LH-nRNP, the latter enriched within the rat liver 70–110 S sucrose-gradient fractions. The antibodies in the two sera had identical specificity, efficiently recognizing the

72/74-kDa protein doublet within rat liver nuclear extracts [17]. These antibodies are, hereafter, considered as one antibody population and are referred to as α -72/74A.

To better analyse the specificity of the α -72/74A antibodies for their respective antigens, 2-D gel electrophoresis was performed on the rat liver 70–110 S sucrose-gradient fractions. As anticipated from our previous study [17], the 72/74-kDa polypeptides represented major constituents of this fraction, which in addition resolved into a series of closely spaced isoforms. This is shown here by the Coomassie Brilliant Blue-stained 2-D gel in Figure 1(A) and by the immunochemical verification with the α -72/74A antibody in Figure 1(B). The 2-D gel analysis revealed the presence of an additional immunoreactive protein species (indicated by an arrow in Figure 1B), which was more basic in nature than the 72/74-kDa protein doublet. Since antibodies, affinity-purified from the 2-D gel-resolved 72/74-kDa polypeptides, were unable to identify this new component (results not shown), we concluded that the latter was reacting with a distinct antibody population in the α -72/74A sera. It is now evident that this protein co-existed in the immunogenic material and that its presence could be detected solely by 2-D resolution of the antigen.

Following the realization that a mixed antibody population existed in the initial α -72/74A sera, additional sera with more restricted antibody specificity were produced. For this purpose, the 74-kDa polypeptide alone, which as seen in Figure 1 was clearly resolved on 2-D gels as an apparent single protein species, was gel-excised and used to immunize guinea pigs. The ability of the new antibody population (labelled α -72/74B) to react exclusively with the pair of 72/74-kDa polypeptides is demonstrated in the immunoblot shown in Figure 1(C). The faint reaction to the species of less than 70 kDa is most likely due to limited

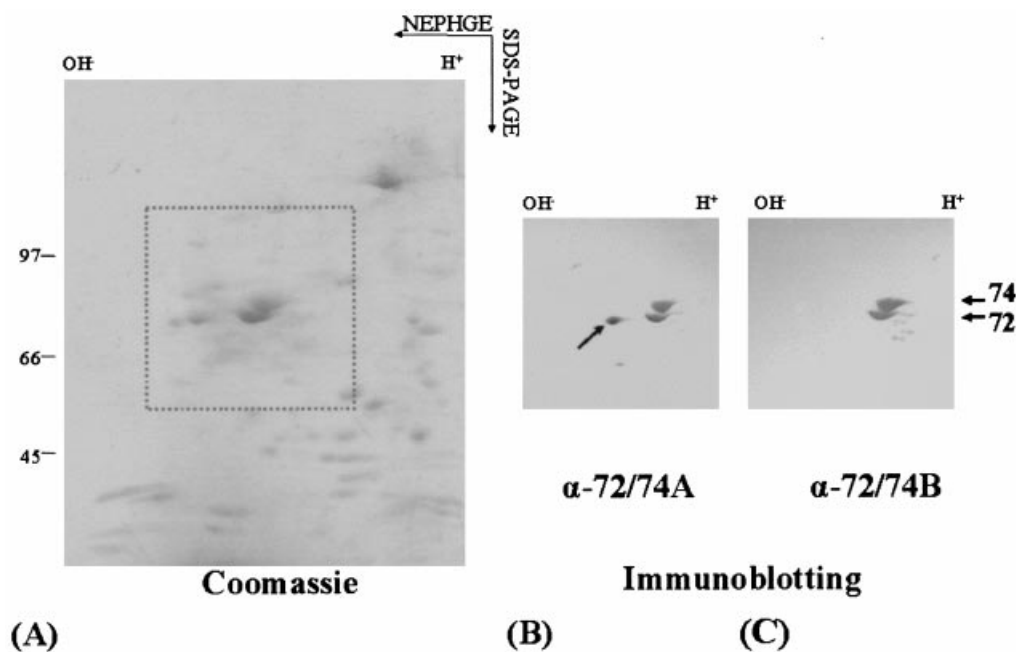


Figure 1 Characterization of antisera with antibody specificity against the 72/74-kDa polypeptides of the LH-nRNP complex

Protein species present within the rat liver 70–110 S nuclear fractions, enriched in the LH-nRNP complex, were resolved by 2-D gel electrophoresis (NEPHGE-SDS/PAGE). In (A), a Coomassie Brilliant Blue-stained gel is shown. Following transfer of a parallel gel to a nitrocellulose membrane, the part of the blot containing the 72/74-kDa protein species (indicated in A by the enclosed square) was immunoblotted using either the α -72/74A (B) or the α -72/74B (C) antisera. Visualization of the immunoreactive species was with the horseradish peroxidase colorimetric system. Arrows point to the 72/74-kDa antigenic components.

protein degradation and became evident when relatively large amounts of the 72/74-kDa polypeptides were applied to the gels. As seen clearly in Figure 1(C), the antibodies made against the 74-kDa protein alone could equally well identify the 72-kDa protein component, confirming our initial observation that the 72/74-kDa proteins are immunologically related protein species [17].

In the present study, the guinea pig sera with either the α -72/74A or the α -72/74B antibody specificity were used interchangeably, as specified in each of the experimental protocols presented.

Amino acid sequence analysis of peptides belonging to the rat 74-kDa polypeptide

From the data outlined above it became apparent that the 2-D resolution of the rat liver 70–110 S sucrose-gradient fractions provided the clean separation of rather high amounts of the 72/74-kDa protein doublet that could be used to probe the nature of the proteins themselves. This was particularly so for the 74-kDa component, which was chosen for further direct investigation. The proteins from 2-D gels were electroblotted on to a PVDF membrane and the exact positioning of the 74-kDa protein species identified immunochemically on a duplicate membrane using the anti-72/74 antibodies. Thereafter, the 74-kDa protein spot was excised from the membrane and subjected to *in situ* digestion with endoproteinase Lys-C. Resolution of the resulting peptides with HPLC and amino acid sequence analysis of four internal peptide fragments then followed (Figure 2A, peptides 1–4). Swiss-Prot comparison revealed that the four peptides shared practically 100% sequence similarity with the human hnRNP M proteins. The latter represent a group of RNA-binding polypeptides, consisting of four known variants (M1–M4), of apparent molecular mass 64–68 kDa and pI 7.8–8.2, which seem to be produced by alternative splicing [7]. The reported sequence of the largest 68-kDa M4 polypeptide has a predicted size of 77 kDa and contains three RBD domains (RBD I and II at the N-terminus and RBD III at the C-terminal part of the protein). The M proteins are Met-rich species, having characteristic Met/Gly- and Met/Arg-rich repeat motifs in between the RBD II and III domains [7]. As shown in Figure 2, the four peptides of the rat 74-kDa protein had an almost perfect alignment with sequences of the human M4 variant. Peptides 1, 2 and 4 were located within the RBD I, II and III domains, respectively, while peptide 3 was within the Met/Gly-rich domain of the M4 protein.

From these findings, the unequivocal identification of the rat 74-kDa polypeptide as a member of the hnRNP M protein family followed. It is also most likely that the second partner of the doublet, the immunologically related 72-kDa polypeptide, belongs to the same protein family (see the Discussion section). We shall refer, hereafter, to the 72/74-kDa polypeptides as 72/74(M) protein species.

Immunoreactivity of anti-hnRNP M antibodies for the rat 72/74(M) polypeptides

The finding that the rat 74-kDa polypeptide was a member of the hnRNP M family prompted us to compare the immunoreactivity of our polyclonal antibodies with that of the monoclonal 1D8 antibody (mo1D8), which has well-established specificity for the human hnRNP M1–M4 variants [7]. Parallel immunoprecipitation reactions were performed on rat liver nuclear extracts with the two antibody populations, followed by 2-D gel resolution of the proteins in the immune pellets and cross-immunoblotting to identify the proteins reacting with each of the

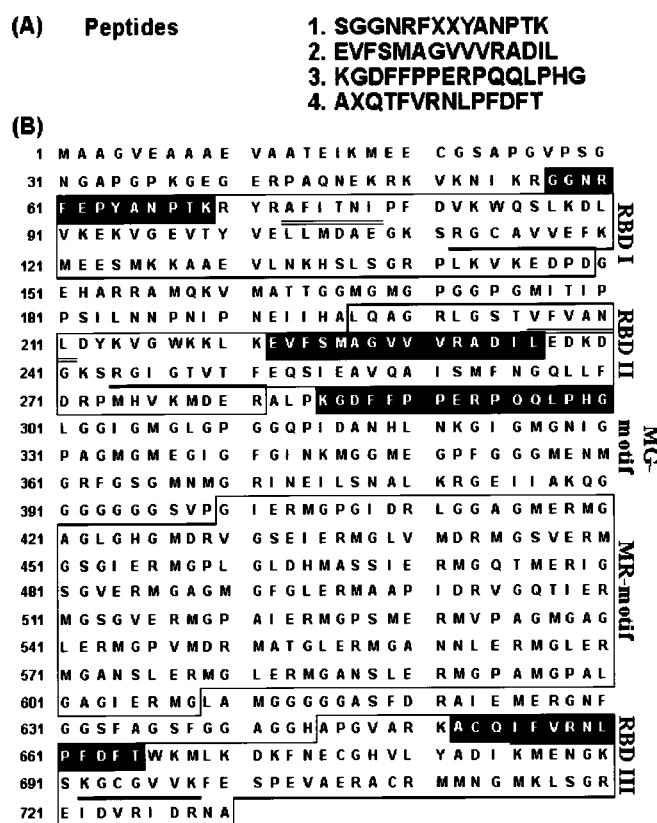


Figure 2 Identification of the rat 74-kDa polypeptide

The 2-D gel-resolved 74-kDa polypeptide was subjected to partial amino acid sequence analysis as described in the Results section. The sequence of the identified internal peptides (1–4) is shown in (A). The regions within the human hnRNP M4 polypeptide that show almost perfect alignment with peptides 1–4 are shown by the black boxes in (B). The motifs corresponding to the three RBD domains (RBD I, II and III), as well as to the MG (Met/Gly)- and MR (Met/Arg)-rich repeat motifs of the hnRNP M protein are also indicated. The heavy and double underlines are used to indicate the RNP-1 and RNP-2 consensus sequences, respectively, within the three RBD domains.

antibody populations. The results obtained are shown in Figure 3. Consistent with our previous findings (see also Figure 1), the polyclonal α -72/74B antibody identified both the 72- and 74-kDa proteins of the doublet in the α -72/74B immunoprecipitate (Figure 3A). However, when an identical membrane was probed with the mo1D8, instead, one reacting species was seen (Figure 3B). This corresponded to the 74-kDa polypeptide, as revealed by the sequential blotting of the same membrane with the α -72/74B antibody (results not shown). Similar analysis of the immunoreactivity of the rat liver proteins present in the mo1D8 immune pellet (Figures 3C and 3D) showed more or less the same picture as that seen in the case of the α -72/74B immunoprecipitate (Figure 3, compare C with A and D with B). The only obvious difference was that a lower amount of the 72-kDa protein was recovered in the mo1D8 compared with the α -72/74B immune pellet. Therefore, by the combined immunoprecipitation and immunoblotting assays, it became clear that the mo1D8 antibody was able to recognize the 74-kDa protein in the α -72/74B immunoprecipitate and vice versa. However, the immunologically related 72-kDa polypeptide could not be identified by the mo1D8 antibody. This result indicated that different epitopes on the hnRNP M proteins were recognized by the two antibodies and that, in particular, the specific

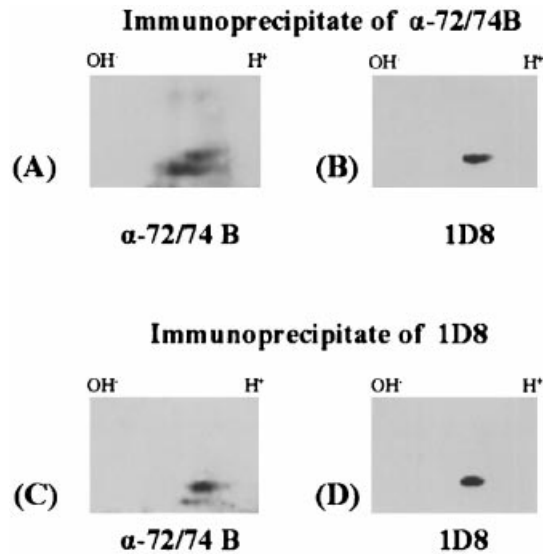


Figure 3 Antigenic properties of the rat 72/74(M) polypeptides

Rat liver nuclear extracts were subjected to parallel immunoprecipitation reactions, in duplicate, using either the α -72/74B antiserum (A and B) or the mo1D8 antibody (C and D). Immunoblotting with the α -72/74B (A, C) and mo1D8 (B, D) antibodies followed the 2-D resolution and transfer to nitrocellulose membranes of the proteins within each of the immune pellets. Immunodetection was with the ECL system.

epitope(s) of mo1D8 on the 74-kDa protein was not shared by the 72-kDa polypeptide.

We have argued previously against the possibility of the 72/74-kDa polypeptides representing members of the hnRNP M family [17]. In the absence of sequencing data, this argument was based on immunoblotting experiments whereby the overall distribution pattern of the 72/74-kDa polypeptides in sucrose-gradient-fractionated rat liver nuclear extracts was investigated using antibodies of different specificity. The initial comparison was made with a monoclonal antibody with specificity for the 72.5/74-kDa polypeptides of the salt-resistant RNP fibrils [22], later shown to correspond to the hnRNP M1/M2 proteins [23]. This antibody (albeit reacting poorly with the rat protein) produced a distinct distribution pattern when compared with the anti-72/74 antibodies; a finding that has now been reproduced with the mo1D8 antibody against hnRNP M1–M4 proteins (results not shown). The differential distribution pattern that was obtained when the polyclonal anti-72/74 and the mo1D8 antibodies were compared with each other could be partly due to the inability of the latter to cross-react with the 72-kDa polypeptide, as shown in the present study. A closer examination of these patterns showed that the mo1D8 antibody recognized proteins in the vicinity of the 72/74-kDa polypeptides that were clearly enriched in the 40–60 S sucrose-gradient fractions (results not shown). This was opposite to the established enrichment of the 72/74-kDa proteins in the 70–110 S sucrose-gradient fractions (see [17]). The parallel application of a monoclonal antibody for the 68-kDa hnRNP L polypeptide [8] revealed a similar distribution profile with that of the mo1D8, in agreement with both L and M proteins being abundant components of immunopurified hnRNP complexes [18].

The apparent differential distribution pattern of the hnRNP M and 72/74(M) polypeptides was investigated further by performing immunoblotting experiments on antigenic material that had been resolved, instead, on 2-D gels. This refers to

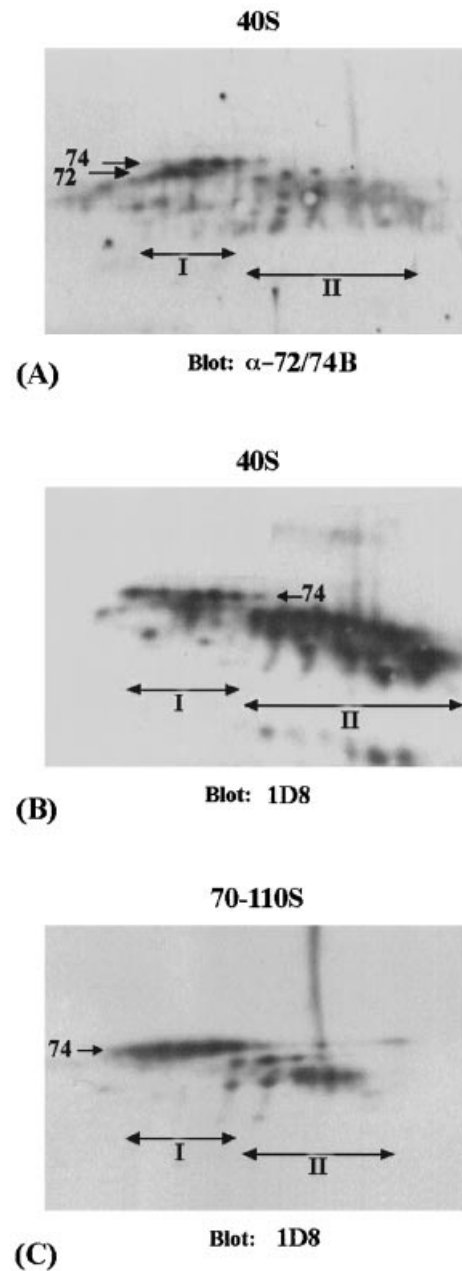


Figure 4 Identification of discrete subsets of polypeptides belonging to the hnRNP M protein family reacting preferably with either the anti-72/74 or the mo1D8 antibodies

Proteins contained within the rat liver 40 S sucrose-gradient fractions were resolved by 2-D gel electrophoresis, transferred to nitrocellulose and blotted using either the α -72/74B antiserum (A) or the mo1D8 antibody (B). The protein species of the 70–110 S sucrose-gradient fractions that are identified by the mo1D8 antibody alone are shown in (C). I and II refer to the protein subsets that are mainly recognized by the α -72/74B and the mo1D8 antibody populations, respectively. Visualization was with the ECL immunodetection system. The mini 2-D gel system of Bio-Rad was applied in this experiment, which did not allow the usual clear separation of the 72/74-kDa protein doublet seen in all other cases with the standard gel system.

fractionated 40 S and 70–110 S sucrose-gradient material, taken to represent the bulk of the hnRNP and LH-nRNP complexes, respectively. The results obtained when identical 40 S fractions were blotted with either the α -72/74B or the mo1D8 antibodies are shown in Figures 4(A) and 4(B), respectively. To facilitate

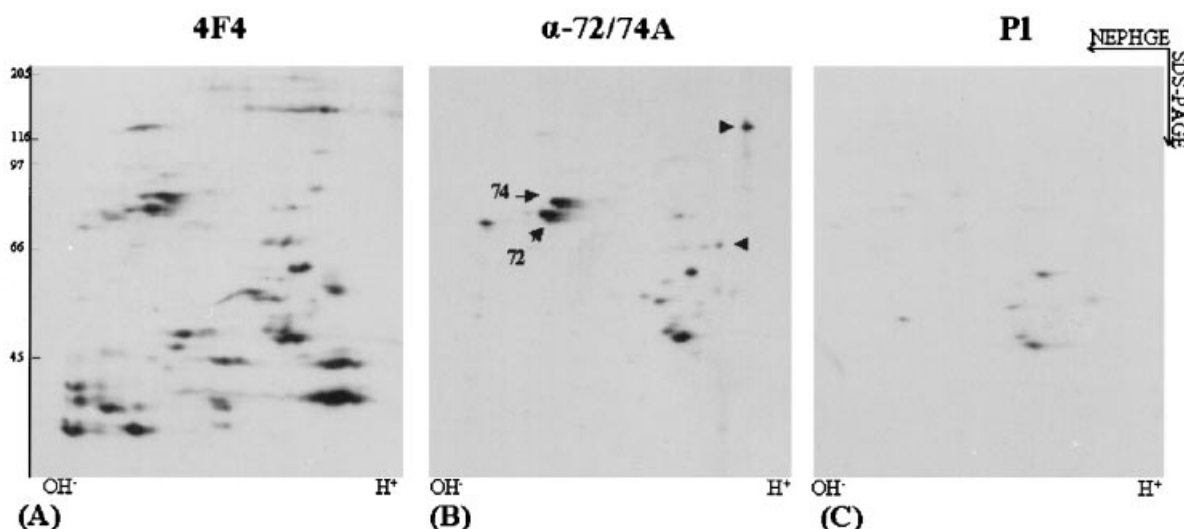


Figure 5 Direct comparison of polypeptides belonging to the hnRNP and LH-nRNP complexes of HeLa cells

Nuclear extracts prepared from [³⁵S]Met-labelled HeLa cells were applied in immunoprecipitation assays whereby the monoclonal 4F4 antibody, used to immunoselect hnRNP complexes [19], was applied in parallel reactions with the α -72/74A and pre-immune (PI) sera. The detection of the labelled protein in the 4F4 (A), the α -72/74A (B) and the PI (C) immune pellets is shown following resolution of the proteins on 2-D gels and subsequent fluorography.

this type of direct comparison and to compensate for the different titres of the two antibody populations, immunoblots are shown with roughly comparable levels of immune reaction for the 74-kDa polypeptide. As discussed above (see Figure 3), the latter, in contrast to the 72-kDa protein, was equally well identified by both the α -72/74B and the mo1D8 antibodies. In Figure 4(A), the major reactivity of the α -72/74B antiserum for the 72/74(M) polypeptides in the 40 S material can be seen. This is despite the fact that in the 40 S fractions these proteins were recovered in rather low amounts (see [17]).

When the reaction of mo1D8 with the same antigenic 40 S material was compared, it became clear that immunoreactivity was mainly against a distinct group of hnRNP M polypeptides (Figure 4B). We refer to the latter as subset II of the M polypeptides, in order to distinguish it from the group that is represented mainly by the 72/74(M) protein species (subset I). The hnRNP M proteins within subset II were found to be highly enriched in the 40 S fraction and appeared to represent at least four different protein species, each resolving into several isoforms. These species, which most likely correspond to the known M1–M4 protein variants, were clearly under-represented in the immunoblot of the α -72/74B (Figure 4A). In Figure 4(C), the immune reaction of the mo1D8 antibody for protein species present in the heavier 70–110 S sucrose-gradient fractions is shown. As seen (Figure 4, compare B and C), for comparable amounts of the 74-kDa polypeptide, the protein species of subset II were now clearly reduced in the 70–110 S fractions compared with the 40 S material. Note should be made of the lack of 72-kDa polypeptide in the immunoblots of either the 40 S or the 70–110 S material, as it cannot be recognized by the mo1D8 antibody. The findings presented in Figure 5, considered together with the distribution patterns discussed above, support the existence of the two discrete subsets of polypeptides belonging to the hnRNP M protein family.

Lastly, it is worth referring to the fact that when the mo1D8 antibody was used to immunoprecipitate the fractionated 40 S material the same picture as shown in Figure 3 for the un-

fractionated nuclear extract was reproduced. Since the bulk of the hnRNP M proteins within subset II were not recovered in the immune precipitate (results not shown), we believe that they were in loose association with the hnRNP complexes, in contrast to the 72/74-kDa proteins in subset I.

Characterization of the 72/74(M) polypeptides within immunoselected HeLa LH-nRNP complexes

A peculiarity of our guinea pig anti 72/74 antibodies (both sera A and B preparations) was their poor performance upon immunoblotting human (HeLa) nuclear extracts; a finding not anticipated from their corresponding good efficiency in rat liver. However, as we have demonstrated previously [17], immunofluorescence and immunoprecipitation assays could be very effectively carried out in HeLa cells. In fact, by immunoprecipitating [³⁵S]Met-labelled HeLa nuclear extracts with α -72/74A antibodies, the presence of a RNP complex having a protein composition identical to the rat LH-nRNP complex was demonstrated [17]. As was pointed out then, LH-nRNP had a rather simple protein composition compared with the hnRNP and snRNP complexes. It contained, in addition to the major 72/74-kDa antigenic proteins, species of 110, 61 and 59 kDa. We have now extended these studies by directly comparing on 2-D gels the protein components of the LH-nRNP and hnRNP complexes. In these experiments, [³⁵S]Met-labelled HeLa nuclear extracts were immunoprecipitated in parallel, using either the guinea pig α -72/74A or pre-immune sera, as well as the monoclonal 4F4 antibody which has been widely used in the literature to identify hnRNP complexes in human cells [19]. In Figure 5(A), the 2-D gel analysis of the 4F4 immunoprecipitate is shown in direct comparison with that of the α -72/74A sera (shown in Figure 5B). It is clear that the picture of the gel in Figure 5(A) accurately reproduced the known pattern of the hnRNP-associated protein components of HeLa cells [18,24]. An overall distinct pattern was obtained from the respective α -72/74A immune pellet, which corresponded to the LH-nRNP complex (Figure 5B). In agreement with our

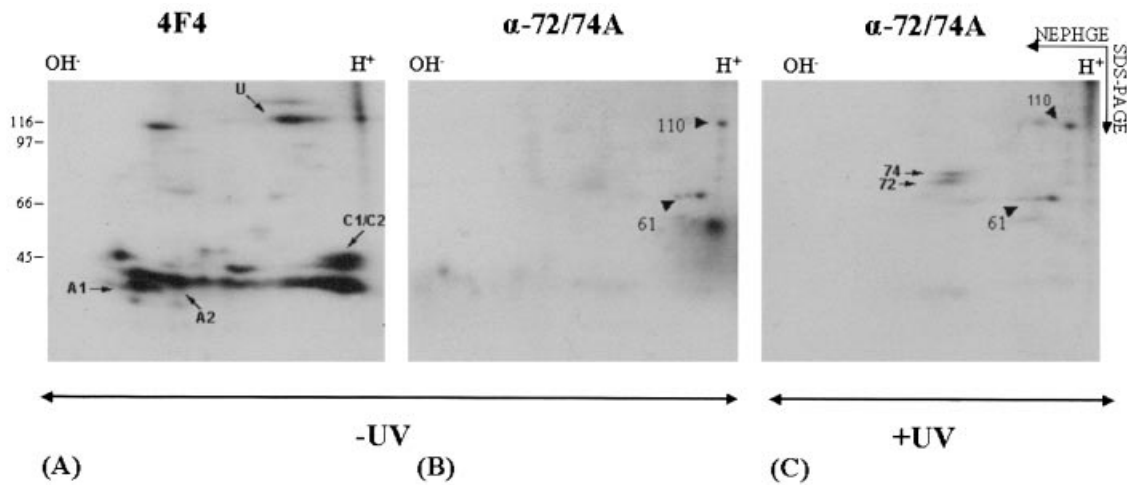


Figure 6 Detection of phosphorylated protein species and RNA-binding polypeptides within immunopurified hnRNP and LH-nRNP complexes of HeLa cells

Nuclear extracts prepared from cells that had been grown in the presence of [32 P]orthophosphate for 17 h, with or without subsequent UV irradiation, were immunoprecipitated using either the 4F4 antibody or the α -72/74A antiserum. Identification of the radiolabelled protein species followed 2-D resolution of the immune pellets and autoradiography. The gels corresponding to the 4F4 and α -72/74A immune pellets from cells that had not received UV irradiation are shown in (A) and (B). The corresponding α -72/74A immune precipitate from UV-irradiated HeLa cells is shown in (C). Arrows point to identifiable polypeptides within the 4F4 and α -72/74A immunoprecipitates.

initial SDS/PAGE analysis [17], the major spots found corresponded to the 72/74-kDa protein doublet. As in the case of rat liver (Figure 1), the HeLa 72/74-kDa protein doublet also resolved into multiple isoforms. When compared with the unspecific proteins found in the control immunoprecipitate (pre-immune serum, shown in Figure 5C), the acidic protein species, marked with arrowheads in Figure 5(B), corresponded to the 110-kDa and most probably to the 61-kDa polypeptides of the LH-nRNP. A protein spot of similar size to the 72-kDa polypeptide but more basic in nature was identified as the protein recognized by the distinct antibody population co-existing in the α -72/74A serum. In accordance with what it was discussed above (see Figure 1), this spot was not seen when the guinea pig α -72/74B serum was used instead (results not shown). Thus the resolution of the hnRNP and LH-nRNP complexes from HeLa cells (shown in Figures 5A and 5B, respectively) verified our previous assumption that these complexes could be taken to represent two overall distinct RNP entities in mammalian cell extracts.

The investigation of the LH-nRNP complex in HeLa cells was extended to include some *in vivo* characteristics of its associated protein components. To this extent, UV irradiation was applied to cells that had been growing overnight in [32 P]orthophosphate-containing medium. As is well established, this protocol allows the UV-mediated indirect labelling of proteins that are in contact with RNA ([21] and references therein). In addition, in the absence of UV irradiation, labelling alone permits the identification of phosphoproteins by the exchange of phosphate groups on the respective protein (as shown in [25]). This type of analysis was, then, combined with subsequent immunoprecipitation of the LH-nRNP or hnRNP complexes (using the α -72/74A and 4F4 antibodies, respectively), followed by 2-D gel resolution of their protein components, as shown in Figure 5. The 2-D electropherograms containing the proteins of the 4F4 and α -72/74A immune precipitates from unirradiated HeLa cells are shown in Figures 6(A) and 6(B), respectively. The relevance of this experimental approach is demonstrated clearly in the case of the 4F4 immunoprecipitate shown in Figure 6(A). The highly phosphorylated hnRNP protein species A1, A2, C1, C2

and U [25] were indeed identified. That the labelling was associated with the presence of phosphorylated protein species was verified by digesting the immune pellets with phosphatases, which resulted in the complete loss of the label (results not shown). In the case of the α -72/74A immune precipitate (shown in Figure 6B), protein spots corresponding to the 110- and 61-kDa LH-nRNP-associated protein components were present. The analysis of the α -72/74A immunoprecipitate originating from labelled cells that had, in addition, received UV irradiation is shown in Figure 6(C). Direct comparison of Figures 6(B) and 6(C) clearly shows that the UV-mediated labelling of protein spots corresponded to the 72/74-kDa polypeptides of the LH-nRNP complex. This indicates the direct contact of the 72/74-kDa polypeptides to the RNA *in vivo*. The presence of the 110- and 61-kDa polypeptides was also apparent on the gel shown in Figure 6(C). Since the intensity of their signal did not increase by UV irradiation (Figure 6, compare B and C) and labelling was sensitive to phosphatase treatment (results not shown), we concluded that these species, in contrast to the 72/74-kDa proteins, were not RNA-binding polypeptides.

DISCUSSION

In the present study, strong evidence was produced to show that the major protein constituents of the LH-nRNP complex, the 72/74-kDa polypeptides, belong to the hnRNP M family. The known members of this family (hnRNP M1–M4) constitute abundant species of hnRNP complexes in human cells [7]. Additional hnRNP M-like proteins have been identified, immunochemically, outside humans, but so far none has been established as an authentic M protein species [26].

It is clear from our findings that the rat 74-kDa protein represents the murine homologue of a human hnRNP M species. Although the limited sequence analysis performed did not allow its assignment to a known M protein variant, it has illustrated the high degree of conservation of members of this family in mammals. Relevant information that relates to the 72-kDa polypeptide of the rat does not yet exist. As shown in this and our

former [17] study, the 72/74-kDa polypeptides represent a protein doublet with identical biochemical and immunochemical properties. The 72-kDa protein species can, thus, be considered as an immunologically novel variant of the hnRNP M family. In this context, it is worth referring to the possible relationship between the 72/74(M) polypeptides and the pair of 72.5/74-kDa protein components of the RNP fibrils [22,23]. Despite their common biochemical features (with respect to size, salt-resistance and association with a RNP complex), they most likely represent related but discrete entities. This assumption relies mainly on the assignment of the 72.5/74-kDa proteins as M1/M2 polypeptides [23], whereas, by all arguments presented above (see also below), the 72/74(M) doublet appeared to correspond to distinct protein variants. Resolution of this issue should, however, await the complete amino acid sequence analysis of the 72/74(M) polypeptides.

We have also provided evidence on the existence of two discrete subsets of the hnRNP M protein family; subset I referring to the 72/74(M) species and subset II to the M1–M4 variants. Additional supportive observations relate to their differential molecular sizes and pI values. Subset II had an apparent molecular size of 64–68 kDa and a pI range of 7.8–8.2 [7]. In contrast, subset I had higher molecular size, and pI comparable with that of the basic hnRNP A2 protein (8.4–8.8; see [5] for references therein). Note should also be made of our reported findings on the immunolocalization of the 72/74-kDa proteins in fixed HeLa cells. These studies revealed the presence of a number of coarse speckles within a rather diffuse nucleoplasmic background [17], which is in contrast to the homogeneous localization of the hnRNP M proteins [7], as is also the case for the hnRNP A1 polypeptide [27].

The identification of the 72/74(M) polypeptides as discrete members of the hnRNP M protein family has increased the number of isoforms belonging to this class of proteins. One can envisage a specialized RNA specificity assigned to each particular isoform, serving a fine-tuning role in RNA-protein recognition, in line with recent reports on other related hnRNP protein families [28,29]. The biological significance of this expanding family of hnRNP proteins is not fully understood today. The limited number of relevant reports refer to the hnRNP M protein participation in RNA splicing [23], as well as to changes related with heat shock [22,23]. This is in contrast to the attention given to other families of hnRNP polypeptides, especially to the group of A/B-type proteins for which a multiplicity of function in the many facets of RNA metabolism has been unravelled (see [15] for a recent review). It is also worth referring to a recent report whereby the identification of over 60 spliceosome-associated polypeptides has been accomplished by the combined application of MS and expressed sequence tag (EST) database searching [30]. This study revealed the presence of hnRNP M protein species amongst the spliceosome-associated components, together with the only other hnRNP polypeptides, A1, A2/B1 and C, for which their role in basic as well as in alternative splicing has been established (reviewed in [5,15]). From the above, the association of members of the hnRNP M family with at least three separate nuclear RNP entities, the hnRNP, the spliceosomal and the LH-nRNP assemblies can be envisaged.

Strikingly similar LH-nRNP complexes have been immunopurified from rat and human nuclear extracts, exhibiting a rather simple protein composition when compared with hnRNP [17]. With respect to its major 72/74-kDa protein species, their high efficiency of labelling by [³⁵S]Met, as well as the UV-induced binding to the RNA *in vivo*, are all in accordance with their current identification as members of the hnRNP M family. The same is true regarding their anticipated functioning in RNA

splicing [23]. We have also shown inhibition of splicing by anti-72/74 antibody addition and/or immunodepletion of HeLa splicing extracts [17]. The nature of the associated polypeptides of 59, 110 and 61 kDa has not been identified further, apart from the observation made in the present study that the 110- and 61-kDa components were phosphoproteins that lacked the ability to bind directly to the RNA. A similar phosphorylation of the 72/74-kDa polypeptides was not detected in our experimental system. Since the hnRNP M polypeptides have been reported to be potentially phosphorylated species [7], it could be that either some phosphatases were still active in our extracts or that the 72/74-kDa proteins of the LH-nRNP complex represent an underphosphorylated subset of the hnRNP M polypeptides. The latter would be in agreement with their observed tight binding to the RNA ([17] and present study).

From all the evidence provided it is clear that LH-nRNP can be obtained as a distinct RNP entity, apart from the bulk of the most abundant hnRNP complexes with which it, nonetheless, shares specific protein and RNA species (present study and [17]). As established, hnRNP complexes represent constituents of the RNA-containing nuclear matrix (see reports [16,31] and references therein). The direct involvement of nuclear matrix components in RNA processing, especially in RNA splicing, has been demonstrated [32,33]. We have also provided evidence to suggest the tight association of the LH-nRNP complex with the nuclear matrix [34]. In this context, we point out the extreme salt-resistance of the 72/74(M) polypeptides [17], which is in contrast to the moderate resistance to salt exhibited by the majority of the hnRNP proteins ([35] and references therein). It is likely that the LH-nRNP complex, via its major protein constituents, the 72/74(M) species, can serve a role in bridging the proteinaceous nuclear matrix with the hnRNP complexes and, possibly, with the other components of the RNA processing machinery, e.g. the spliceosome. Further studies aiming at defining such envisaged interactions will contribute towards understanding its exact functioning in RNA metabolism.

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