Identification of S1 proteins B2, C1 and D1 as AUF1 isoforms and their major role as heterogeneous nuclear ribonucleoprotein proteins

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AUF1 (A + U-rich RNA binding factor) participates in the rapid decay of mRNAs in the cytoplasm. It is sometimes called heterogeneous nuclear ribonucleoprotein (hnRNP) D0; however, evidence for its characterization as an hnRNP protein has been scarce. S1 proteins A–D are those selectively extracted at pH 4.9 from isolated nuclei pretreated with either RNase A or DNase I. In the present study we identified S1 ('first supernatant') proteins B2, C1 and D1 with p45, p40 and p37 AUF1s respectively, by microsequencing and product analysis of transfected cDNAs. We found, further, that more than 96 % of the S1 proteins occurred in the nucleus, and localized largely in RNase-sensitive structures. B2 was confined in the nucleus and C1 directly bound to heterogeneous nuclear RNAs (hnRNAs). These B2 and C1 proteins formed hnRNP structures responsible for the 33 S, and, to lesser extent, the 40 S particles, which were liberated upon mild nucleolytic cleavage. On the other hand, D1 and the remainder of C1 were associated with nuclease-hypersensitive sites of hnRNAs, and comprised the major cytoplasmic AUF1s that may be involved in mRNA decay. Two-dimensional immunoblotting resolved each S1 isoform into up to six spots or more, and suggested that the previous uncertain relationship of hnRNP D0 and hnRNP D is resolved in terms of charge differences and differential splicing arising from one gene. The present results thus indicate that S1 proteins B2, C1 and D1 are identical with AUF1 proteins, but largely occur as hnRNP proteins in the nucleus. That hnRNP D0 is indeed an hnRNP protein was verified.

Key words: heterogeneous nuclear RNA (hnRNA), heterogeneous nuclear ribonucleoprotein D (hnRNP D), hnRNP D0, multi-functional protein, splicing isoform.

INTRODUCTION

S1 proteins A–D occur at sites sensitive to RNase as well as DNase and are extracted at pH 4.9 from the reaction supernatants of nuclei mildly treated with either enzyme [1–3]. S1 ('first supernatant') proteins consist of eight proteins: A1 (migrating on SDS/PAGE with an apparent molecular mass of 74.5 kDa), A2 (69.5 kDa), B1 (47.4 kDa), B2 (46.5 kDa), C1 (43.9 kDa), C2 (42.8 kDa), D1 (40.8 kDa) and D2 (39.4 kDa). A rabbit polyclonal antibody R2 raised against rat S1 protein B specifically reacted with proteins B2, C1 and D1 [4,5]. These proteins are found in the euchromatin bordering the heterochromatic area [5] where RNA polymerase II transcription actively takes place (reviewed in [6]). In the present study we elucidated the biological significance of the S1 proteins B2, C1 and D1.

Heterogeneous nuclear RNAs (hnRNAs) are RNA polymerase II transcripts found in the cell nucleus, many of which are processed to functional mRNAs. hnRNAs are associated with various proteins and form heterogeneous ribonucleoprotein complexes [heterogeneous nuclear ribonucleoproteins (hnRNPs)] that sediment between 40 and 250 S [7]. By mild *in vitro* action of endogenous or exogenous RNase(s), hnRNPs are fragmented, producing 40 S particles, which may represent the basic repeat structures in hnRNPs [8,9]. The 40 S particles contain an RNA fragment of about 700 nucleotides and multiple copies of hnRNP A, B and C as core proteins [9]. In addition to the transcript packaging, hnRNP proteins are involved in various reactions: transcriptional regulation, splicing and polyadenylation of pre-mRNAs, and export, turnover and translational silencing of mRNAs (reviewed in [10,11]). In fact, we recently characterized S1 proteins C2 and D2 as novel hnRNP proteins [12], and identified them with transcription factors, CArG-box binding factor-A ('CBF-A') and its splicing isoform [13–15].

In the cytoplasm, AUF1 (A + U-rich RNA binding factor) is associated with AU-rich elements (AREs) within 3′ untranslated regions (3′ UTRs) of labile mammalian mRNAs and is involved in their rapid degradation [16–18]. It binds to AREs probably through other proteins such as lactate dehydrogenase [19]. AUF1 also acts as a transcriptional regulator. It binds and activates various genes such as the complement receptor 2 (CR 2) gene [20], Epstein–Barr-virus C promoter [21], Ig heavy-chain gene [22] and so on. AUF1 suppresses gene expression too [23]. Participation of AUF1 in telomere maintenance and splicing reactions has been also suggested [24–26]. AUF1 consists of four isoforms, p45, p42, p40 and p37, generated by alternative splicing of mRNA precursor [27]. p40 and p37 are major cytoplasmic AUF1s [28], and continuously shuttle between the nucleus and the cytoplasm [23].

Abbreviations used: ARE, AU-rich element; AUF1, A + U-rich RNA binding factor; hnRNA, heterogeneous nuclear RNA; hnRNP, heterogeneous nuclear ribonucleoprotein; UTR, untranslated region; RSB, reticulocyte standard buffer; S1 proteins, a name originally given to the proteins that were selectively extracted at pH 4.9 from the first supernatant of nuclei previously digested with DNase I.

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AUF1 is also known as hnRNP D0. The identity of AUF1 as hnRNP D0 has been demonstrated by cDNA cloning of AUF1 [27,29]. In contrast with the extensive studies on its involvement in the cytoplasmic mRNA turnover, studies characterizing it as an hnRNP protein have been scarce. hnRNP D0 proteins were cloned for the proteins that preferentially bind to the sequence UUAG, which is present in pre-mRNA 3′ splice sites, as well as to the sequence TTAG of human telomeres. hnRNP D0 proteins were shown to react to an anti-(hnRNP D) monoclonal antibody, namely 5B9, and have a similar, but different, mobility to those of hnRNP D proteins in two-dimensional gel electrophoresis. Hence they were designated 'hnRNP D0' [24,26]. The name 'hnRNP D' (D1 and D2) had been given to two proteins among various proteins that were co-immunoprecipitated with an antibody against hnRNP C protein [30].

In the present paper we report that S1 proteins B2, C1 and D1 are identified with AUF1 isoforms and exist mostly as hnRNP proteins in the nucleus. The results also resolve the longstanding uncertainty about the relationship between hnRNP D0 and hnRNP D in terms of charge and splicing isoforms arising from one gene.

EXPERIMENTAL

Protein preparation and immunoblotting

Reference S1 proteins were prepared from rat liver nuclei by the DNase I digestion method described previously [1,12]. Briefly, isolated nuclei were washed thoroughly in 0.3 M sucrose/3 mM $MgCl_2$/12 mM Tris/HCl (pH 7.4)/0.2 mM PMSF and digested with DNase I (2.5 μg/ml) at 30 °C for 8 min. The mixture was adjusted to pH 4.9 with 0.1 M Na_2EDTA to a final concentration of 5 mM, and centrifuged twice for 10 min each time, the first time at 3000 ***g***, followed by re-centrifugation of the supernatant at 20 000 ***g***. Proteins in the supernatant were collected, and dissolved in SDS sample buffer [62.5 mM Tris/HCl (pH 6.8)/10 % (v/v) glycerol/2 % (w/v) SDS/1 % (v/v) β-mercaptoethanol].

Total proteins from cultured cells were obtained from the cells rinsed with PBS and solubilized in 0.3 ml of SDS sample buffer/85-mm-diameter plate. The viscous samples were sonicated [Branson sonifier 250 (Branson Sonic Power Co., Danbury, CT, U.S.A.); the output setting was 1.5, with a microtip giving ten 3 s bursts on ice].

Total proteins from various mouse tissues were prepared as follows: finely minced tissue ($\approx$ 0.1 g) was solubilized by Dounce homogenization in 20 vol. of SDS sample buffer. The preparations were sonicated.

Protein samples from the above three preparations were heated at 95 °C for 5 min and analysed by immunoblotting. When needed, blots were first stained with Coomassie Brilliant Blue. For immunostaining using an ECL® detection kit (RPN 2106; Amersham Biosciences), the blots were washed in methanol to remove Coomassie Brilliant Blue, then in TBS [10 mM Tris/HCl (pH 7.5)/0.15 M NaCl]. Reprobing was done according to the manufacturer's instructions. Molecular masses were determined using protein marker kits (#7702S and 7708S; New England BioLabs, Beverly, MA, U.S.A.).

Two-dimensional gel electrophoresis

For the first-dimension isoelectric focusing, precast gels (Immobiline DryStrip; pH 3.0–10.5; 11 cm; Amersham Biosciences) were swollen with 240 μl of S1-protein-containing buffer (8 M urea/0.5 % Triton X-100/0.075 % dithiothreitol/0.2 % Ampholine pH 3.5–9.5) for 15 h. Electrophoresis was done on a Multiphor II (Amersham Biosciences) and then on second-dimension SDS gels (9 % gel; 1 mm $\times$ 150 mm $\times$ 150 mm) according to the manufacturer's protocols.

DNA determination and copy number estimation

Sample DNA was isolated by the standard phenol/chloroform method, including prior digestions with proteinase K (100 μg/ml for 3 h), and RNase A (20 μg/ml for 2 h) and was determined by assuming that an A_{260} of 1.0 corresponds to 40 μg of DNA/ml in water. S1 proteins in subcellular fractions and in various tissues were estimated by immunoblotting using anti-(S1 protein) antibody R2 with reference rat liver S1 proteins as standards. The amount of each reference S1 protein was assumed to be proportional to the band intensity stained with Coomassie Brilliant Blue. The reactivity of antibody R2 towards S1 proteins B2, C1 and D1 differed: at an equal amount they exhibited a relative band intensity 0.75:1.0:0.30. These degrees of reactivity were taken into account in the estimation of copy numbers. Also used for the estimation were molecular masses (38.2, 32.6 and 30.5 kDa for p45, p40 and p37 AUF1 isoforms), a DNA content 3×10^9 bp for a single cell, and a mass of 650 Da for 1 bp.

Cells, immunofluorescence staining and transfection

Rat liver epithelial cells (ARL) were grown in William's E medium, and human hepatoma (HLE) cells, rat Morris hepatoma (HTC) cells, monkey kidney (Cos) cells and hamster kidney (HaK) cells were grown in Dulbecco's modified Eagle's medium ('DMEM') at 37 °C in a CO_2 incubator. The media were supplemented with 5 % (v/v) fetal calf serum. ARL cells were stained as described [12] using antibody R2 and a secondary rhodamine (tetramethylrhodamine β-isothiocyanate)-conjugated anti-rabbit IgG antibody (Sigma, T-7028). Expression plasmids of human p45, p42, p40 and p37 AUF1 cDNAs were constructed by digestion of the plasmids pGBT9-p45, -p42, -p40 and -p37 AUF1 cDNAs [23] respectively with *Eco*RI and *Bam*HI, and ligation of the inserts to the *Eco*RI and *Bam*HI sites of pcDNA3.1 (Invitrogen). Cells were grown in 3.5-cm-diameter dishes at about 80 % confluency, rinsed and transfected with liposomes formed with plasmid (1 μg), 4 μl of LIPOFECTAMINE™ and 6 μl of PLUS™ reagent (GIBCO BRL catalogue no. 10964-013) in 1 ml of serum-free medium. The transfected cells were incubated for 64 h, with an addition of complete medium (3 ml) at 4 h and a replacement with fresh medium at 20 h [12]. Total proteins were prepared as described above.

Labelling of hnRNA, immunoprecipitation and UV cross-linking

Labelling of hnRNAs with [5,6-^{3}H]uridine in ARL cells in culture, partial purification of [^{3}H]hnRNPs and immunoprecipitation using Protein A–Sepharose beads (4 Fast Flow; Amersham Biosciences) were performed as described in [12]. Immunoprecipitated [^{3}H]hnRNAs were subjected to Northern blotting [12]. The blot was analysed on an image analyser (Bas-2000 II; Fuji Film, Tokyo, Japan). UV cross-linking of proteins to bound [^{3}H]hnRNAs in ARL cells, and analysis of [^{3}H]uridine-conjugated S1 proteins, were done as described in [12].

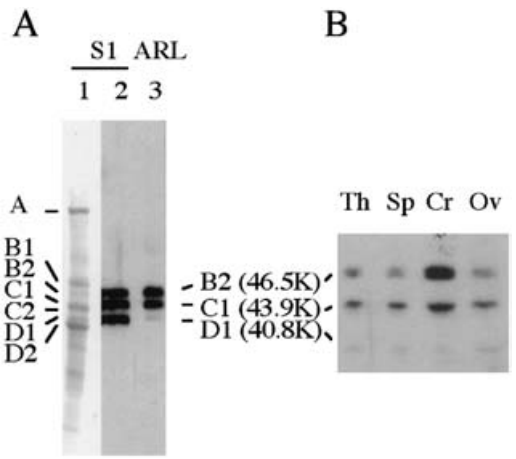


Figure 1 S1 proteins B2, C1 and D1, and the specificity of anti-(S1 protein) antibody R2

(**A**) Rat liver S1 proteins prepared by the DNase I digestion method (lanes 1 and 2), and total proteins from ARL cells (lane 3) were run on an SDS-containing gel (6 cm long, 11 % acrylamide) and blotted on to a PVDF membrane. The blot was cut into strips and stained with Coomassie Brilliant Blue (lane 1), or with anti-(S1 protein) antibody R2 and a secondary horseradish peroxidase-conjugated antibody (lanes 2 and 3). Apparent molecular masses were estimated with protein marker kits. (**B**) Total proteins from various tissues of the mouse were immunoblotted as described above. The SDS-containing gel (9.5 % acrylamide) was 18 cm long. Abbreviations: Th, thymus; Sp, spleen; Cr, cerebrum; Ov, ovary.

Sucrose-gradient centrifugation and 40 S hnRNP particles

hnRNPs were extracted three times from isolated nuclei from rat liver or HTC cells with 0.6 ml portions of a solution containing 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.4 mM PMSF, 10 μg/ml each of pepstatin A and leupeptin hemisulphate, 100 kallikrein-inhibitory units/ml aprotinin [RSB (reticulocyte standard buffer)/proteasin solution]. The second and third extracts were combined, treated with or without RNase A or DNase I (50 μg/ml) at 20 °C for 15 min, and 0.5 ml of each was analysed on 5–20 % (w/v) sucrose density gradients [12]. The 40 S particles were prepared by incubation of nuclei from rat liver or ARL cells for a total of 1.5 h at 4 °C to convert hnRNPs to 40 S particles, and the supernatants containing the released 40 S particles were run on 5–20 % sucrose gradients [12].

Protein sequencing

This was carried out as described in [12].

RESULTS

S1 proteins and polyclonal anti-S1 protein antibody R2

Figure 1(A) shows rat liver S1 proteins, which were prepared by the DNase I digestion method, where S1 proteins were extracted at pH 4.9 from nuclei previously digested with DNase I. Apparent molecular masses of B2, C1 and D1 were 46 500, 43 900 and 40 800 Da respectively (lane 1). The polyclonal anti-(S1 protein) antibody R2 used in the present study recognized S1 proteins B2, C1 and D1 (lane 2). Its high specificity was shown with total proteins from ARL cells (lane 3). S1 proteins prepared by the DNase I digestion method contained a significant amount of protein D1 (lane 2); however, its abundance in whole cells was usually low (lane 3). This lower cellular abundance of protein D1 was common to all examined cultured cells (results not shown) and various tissues of the mouse (Figure 1B).

S1 proteins are identified with AUF1 proteins

S1 proteins were resolved by longer SDS gels (18 cm) for microsequencing. They were blotted to PVDF membrane, and the proteins B2, C1 and D1 on excised strips were digested with *Staphylococcus aureus* V8 protease or lysylendopeptidase (*Achromobacter* protease I). The peptides produced from each S1 protein exhibited similar elution profiles on HPLC chromatograms (results not shown). A total of nine selected peptides were sequenced for protein C1, five peptides for protein D1 and two for protein B2 (Figure 2); all peptide sequences coincided with the sequence of p40 AUF1 protein, also called hnRNP D0 ([26,29]; GenBank® accession numbers for rat AUF1 sequences: ABO46615–ABO46618).

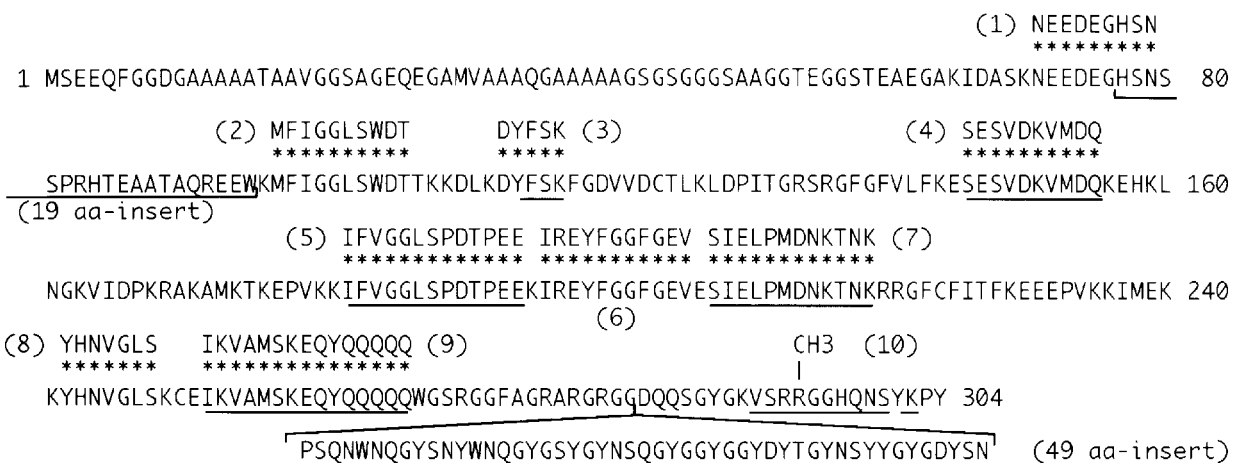


Figure 2 Sequence analysis of S1 proteins: identity with AUF1 proteins

Amino acid sequences were determined for the peptides obtained by digestion of S1 proteins with lysylendopeptidase (peptides 1–3, 5, 6, 8 and 10) or with *S. aureus* V8 protease (peptides 4, 7 and 9). The peptide sequences of protein C1 are aligned with the p40 AUF1 sequence. Asterisks (*) indicate identical amino acids. AUF1 has four isoforms – p37, p40, p42 and p45 – arising from alternative splicings responsible for the N-terminal 19- and C-terminal 49-amino-acid inserts. p40 contains the 19-amino-acid insert (amino acids 77–95), but not the C-terminal 49 amino acids, which are shown on the bottom line. Underlined peptide sequences 5, 7, 9 and 10, part of the sequence of peptide 3, and sequences 4 and 9, were also determined for proteins D1 and B2 respectively. The tyrosine residue (Y) in peptide 10 was not determined unambiguously. The second arginine (R) residue in peptide 10 may be methylated.

AUF1 proteins comprise four isoforms (p37, p40, p42 and p45 [27]), depending on the presence or absence of the N-terminal 19- and C-terminal 49-amino-acid inserts resulting from alternative splicings (Figure 2). The S1 protein C1 yielded a peptide (peptide 1 in Figure 2), which overlapped the N-terminal 19-amino-acid insert found in AUF1 p40 and p45. This, and the molecular sizes of the proteins, suggest that the protein C1 corresponds to p40 AUF1. The smaller D1 then corresponds to p37 AUF1 isoform (with no insert), and the larger B2 to p42 AUF1 (with a 49-amino-acid insert) or p45 AUF1 (with both inserts).

The second arginine residue in peptide 10 was not determined. Its retention time on the sequencer was, however, close to that of monomethyl- and dimethyl-arginine [31,32]. Since the amino acid corresponding to this residue in AUF1 is arginine, it was assumed to be arginine modified with methyl group(s).

Evidence for the identity of S1 proteins with AUF1 isoforms

To examine the above identification, cDNAs of human AUF1 p45, p42, p40 and p37 were transfected into human HLE cells, and total cellular proteins were resolved by SDS/PAGE (Figure 3A, left panel) and analysed by immunoblotting. As expected, the S1 protein-specific antibody R2 strongly recognized AUF1 isoforms expressed from the cDNAs (right panel, lanes 5–8). In accordance with their molecular sizes, the mobility of cDNA products increased in the order: p45 < p42 < p40 < p37. The endogenous S1 proteins were present at lower levels (lanes 3 and 4), thereby serving as good internal references. Importantly, the mobility of the reference rat liver S1 proteins B2, C1 and D1 (lanes 1 and 2), as well as of the endogenous S1 proteins B2, C1, and D1 of HLE cells (lanes 3 and 4), perfectly coincided with that of the p45, p40 or p37 AUF1 protein expressed from the corresponding cDNAs respectively. Thus the specific recognition of AUF1s by antibody R2 and the perfect coincidence of gel mobility of corresponding S1 and AUF1 isoforms not only confirmed the identity of S1 proteins C1 and D1 with p40 and p37 AUF1s respectively, but also assigned S1 protein B2, not to p42 AUF1, but to p45 AUF1. In addition, Figure 3(B) demonstrates a more distinct resolution of p40 and p42 AUF1 isoforms using a long (18 cm) gel.

The perfect mobility coincidence of the endogenous S1 proteins with corresponding AUF1 cDNA products, as well as the consistent mobility order between these isoforms, were also obtained with all other cell types examined: rat hepatoma HTC cells, (simian-virus-40 T-antigen-containing) monkey Cos cells and hamster HaK kidney cells (results not shown). Hence, although S1/AUF1 isoforms undergo post-synthetic modifications as discussed below, and could alter their electrophoretic mobility, they show the consistent gel-mobility order irrespective of different cell types where modifications could occur to various extents.

From the amino acid sequencing (Figure 2) and the transfection experiments (Figure 3A), we concluded that S1 proteins B2, C1 and D1 are p45, p40 and p37 AUF1s respectively.

S1 proteins (AUF1s) in various tissues: low expression of p42 AUF1

Under the present conditions, p42 AUF1, which contains the C-terminal 49-amino-acid insert, was hardly detected by SDS/PAGE in rat liver S1 proteins or by immunoblotting with antibody R2 in total proteins of various cultured cells. Likewise, in whole-tissue extracts of the mouse, p42 AUF1 was not detected (Figure 3C). These results indicate that the expression of p42 AUF1 is generally significantly low as compared with other isoform proteins. In addition, among S1 proteins, B2

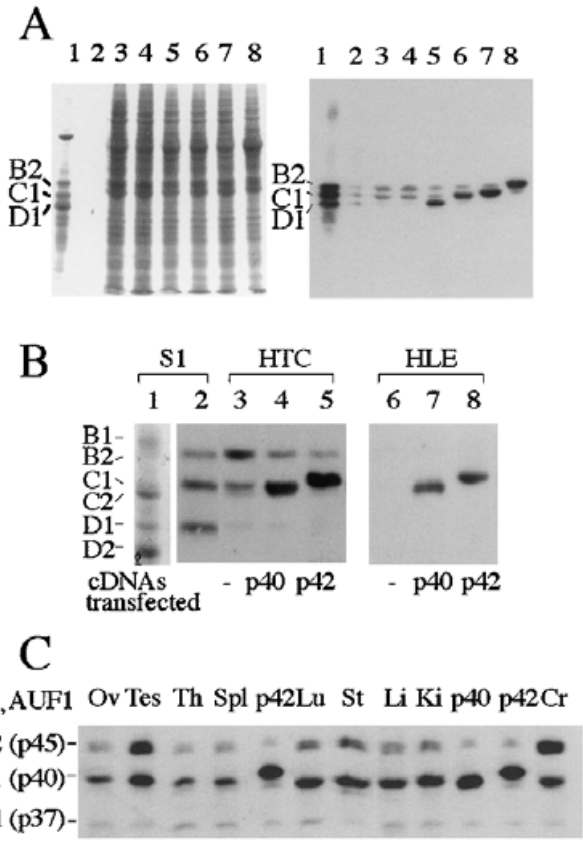


Figure 3 Evidence for the identity of S1 proteins B2, C1 and D1 with p45, p40 and p37 AUF1 isoforms: immunoblotting using antibody R2

(**A**) HLE cells were transfected with liposomes containing the expression plasmid of human AUF1 cDNA. After 64 h, total proteins were analysed (left panel: the blot stained with Coomassie Brilliant Blue; right panel, the same blot probed with antibody R2). Samples are reference rat liver S1 proteins (lanes 1 and 2) and total extracts of the cells treated with no liposome (lane 3), liposomes alone (lane 4) and liposomes containing p37, p40, p42 or p45 AUF1 cDNA, respectively (lanes 5–8). (**B**) Identity of S1 protein C1 with p40 AUF1. HTC and HLE cells were transfected with liposomes alone (lanes 3 and 6), or liposomes containing p40 (lanes 4 and 7) or p42 (lanes 5 and 8) AUF1 cDNA. Total cell extracts were analysed on an 18-cm-long SDS-containing 10 %-(w/v)-polyacrylamide gel. Reference rat liver S1 proteins were stained with Coomassie Brilliant Blue (lane 1) or with antibody R2 (lane 2) respectively. (**C**) S1/AUF1 proteins in various mouse tissues. Total proteins from various tissues were separated on an 18-cm-long gel. Abbreviations: Ov, ovary; Tes, testis; Th, thymus; Spl, spleen; Lu, lung; St, stomach; Li, liver; Ki, kidney; Cr, cerebrum. p40 and p42, total proteins from HTC cells transfected with p40 and p42 AUF1 cDNA respectively. Tissue proteins equivalent to 1.8 μg of DNA were analysed, except for the samples from cerebrum and testis (equivalent to 0.36 and 0.9 μg of DNA respectively).

(p45 AUF1) and C1 (p40 AUF1) were more expressed than D1 (p37 AUF1), and the cerebrum and testis especially expressed B2, at significantly higher levels than did other tissues. For example, they expressed 20 and 8 times more B2 than the liver respectively. It seems that the exon coding the N-terminal 19-amino-acid insert is preferentially retained in AUF1 mRNA, thereby producing proteins B2 and C1 (Figure 2). Moreover, in the cerebrum and testis, the exon for the C-terminal 49-amino-acid insert is further included, and produces more B2 than C1.

Localization of S1 proteins in the rat liver cells

S1 proteins existed predominantly in the nucleus, or more precisely in the extranucleolar region of the nucleus [5]. This was shown here by immunofluorescence staining of ARL cells using antibody R2. Although the signals were weak, cytoplasmic

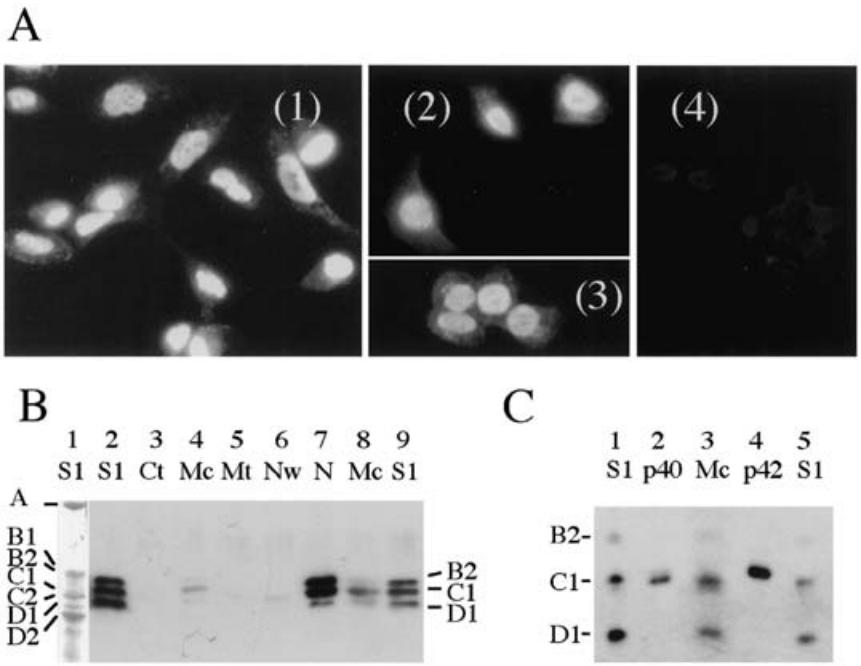


Figure 4 Cellular localization of S1 proteins B2, C1 and D1

(**A**) Immunofluorescence staining. ARL cells were stained with antibody R2 (panels 1, 2 and 3) or with a control preimmune serum (panel 4), followed by a secondary FITC-conjugated anti-rabbit IgG antibody. (**B**) Subcellular fractions from rat liver were analysed on a mini SDS-containing 9.5 % polyacrylamide gel. Blotted proteins were stained with Coomassie Brilliant Blue (lane 1) or with antibody R2 (lanes 2–9). Lanes 1, 2 and 9, reference rat liver S1 proteins (S1); lanes 3–8, cytosol (Ct), microsomes (Mc), mitochondria (Mt), nuclear wash (Nw), washed nuclei (N) and microsomes respectively. A 20 μg of protein was loaded, except for 10 and 50 μg was used for lane 7 (nuclei) and lane 8 (microsomes) respectively. (**C**) SDS/PAGE on an 18-cm-long 10 % polyacrylamide gel. Lanes 1 and 5, reference S1 proteins (S1); lane 3, microsomal fraction (Mc); lanes 2 and 4, total proteins from HLE cells transfected with p40 and p42 AUF1 cDNAs respectively.

staining was apparent when the results with a control pre-immune serum were compared (Figure 4A, panels 1–3 and 4).

Consistent with the strong nuclear immunofluorescence staining, cell fractionation of rat liver indicated that most S1 proteins (> 96 %) were present in the nuclear fraction (Figure 4B, lane 7). In addition, the microsomes and cytosol contained 2.4 and 1.6 % of cellular S1 proteins, respectively, which exhibited similar electrophoretic patterns, shown here with the microsomal fraction (Figure 4B, lane 8). Noticeably, these cytoplasmic S1 proteins are mostly C1 and D1 (p40 and p37 AUF1s) (Figure 4C, lane 3). Copy numbers of rat liver S1 proteins B2, C1 and D1 were in a ratio 100:53:29 in the nucleus, and 0.58:3.6:3.4 in the cytoplasm respectively (the values represent relative abundance of each S1 protein in liver cells). The results are consistent with the observed cytoplasmic occurrence of p40 and p37 AUF1s [23]. It is noteworthy, however, that the nucleus contained most (> 96 %) of the cellular S1/AUF1 proteins.

Nuclear S1 proteins

For further characterization of nuclear S1 proteins, they were extracted from isolated nuclei in a soluble form with a low salt (10 mM NaCl) solution (Figure 5). After the first washing, S1 proteins were extracted gradually, reaching a total 49 % of nuclear S1 proteins in five washings (Figure 5B). An extrapolation predicted about 80 % of nuclear S1 proteins to be extracted in ten washings (results not shown). In contrast with the yield, which decreased from the third washing (Figure 5C, white bars), the S1 protein concentration increased with extraction number (Figure 5C, thin grey bars). S1 proteins remaining in the nuclei were solubilized with high-salt solutions, and completely at 0.6 M NaCl (Figure 5B); 1.0 and 2.0 M NaCl buffer solutions no longer extracted any S1 proteins.

RNase A treatment (50 μg/ml at 30 °C for 15 min) of isolated rat liver nuclei liberated 81, 77 and 99 % of nuclear S1 proteins B2, C1 and D1 respectively. Successive DNase I digestion of the nuclei further liberated 6, 10 and 1 % of them, leaving 13 % each of proteins B2 and C1 in the nuclear residues. These results suggested that nuclear S1 proteins were largely associated with RNase-sensitive structures. The almost complete liberation of protein D1 with RNase A suggests its exclusive association with RNAs in the nucleus.

S1/AUF1 proteins constitute hnRNP complexes

The results of centrifugation on sucrose density gradients of low-salt nuclear extracts from rat hepatoma HTC cells are shown in Figure 6(A). S1 proteins B2 and C1 were spread continuously from top to bottom on the gradients. The fast-sedimenting complexes were up to 200 S or larger. When the nuclear extracts were previously treated with RNase A, all S1 proteins were now recovered in the top fractions. In contrast, DNase I had no effect. The same results were obtained with rat liver nuclear extracts also (results not shown). These results suggested that the extracted S1 proteins came from hnRNP complexes. Protein D1 (p37 AUF1) was also present in large hnRNP complexes up to 200 S. However, it was recovered mostly near the top of the gradients (fractions 12–16, Figure 6A); most of the protein D1 seemed to be associated with nuclease-hypersensitive sites and was liberated by endogenous nuclease(s), particularly under the present conditions including an incubation (20 °C, 15 min) prior to the centrifugation.

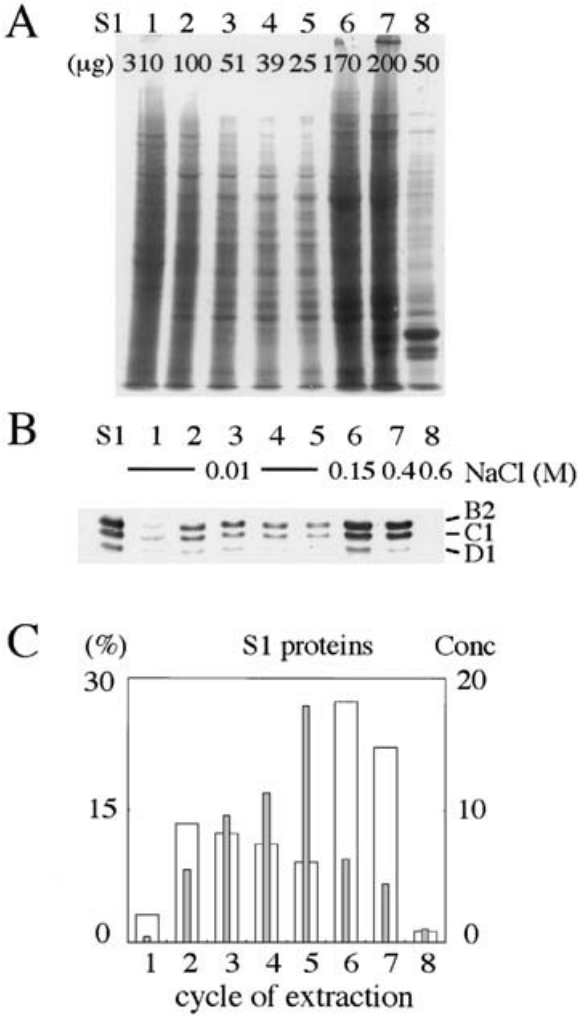


Figure 5 Nuclear S1 proteins

Proteins were successively extracted from rat liver nuclei, firstly five times with 10 mM NaCl in RSB/proteasin solution (lanes 1–5), then in a stepwise manner at 0.15, 0.4 and 0.6 M NaCl (lanes 6–8). They were separated by SDS/PAGE and blotted. The blot was stained with Coomassie Brilliant Blue (**A**), then with antibody R2 (**B**). Amounts of extracted proteins (in μg) starting from 1 g of liver are indicated on each lane in (**A**). S1, reference S1 proteins. (**C**) White bars represent the yield of S1 proteins (% of total) determined by densitometric measurement of the gel in (**B**). Thin grey bars, S1 protein concentration in arbitrary units (% of nuclear S1 proteins/μg of extracted proteins).

The association of S1 proteins with hnRNAs was verified. In this experiment, ARL cells were incubated with [^{3}H]uridine, and the nuclear soluble fraction was immunoprecipitated with antibody R2. The precipitated [^{3}H]RNAs were larger than 400 nucleotides (nt) to up to 30 000 nt, and resolved entirely as a smear on electrophoresis (Figure 6B, lane 3). In contrast, a preimmune serum precipitated RNAs little (lane 2). An association of S1 proteins with RNA polymerase III transcripts is unlikely, because these RNAs, including their precursors, are smaller than 400 nt. Similarly, S1 protein association with pre-rRNPs is unlikely too, because pre-rRNPs are 40–90 S [33,34] and should appear as discrete peaks over the fractions 10–14 on the gradient in Figure 6(A). From these results we concluded that the S1 proteins in the soluble nucleoplasm are in association with hnRNP complexes.

Direct binding of S1 proteins to hnRNAs

p37 and p40 AUF1s (=S1 proteins D1 and C1) bind to overlapping (AUUUA) repeats with high affinities [35] and more strongly to AUUUUUUUA [36]. They also bind to poly(U) [37]. Furthermore, the cytoplasmic p37 and p40 AUF1s have been UV-cross-linked to [^{32}P]uridylate-labelled ARE in an *in vitro* binding experiment [38]. Accordingly, nuclear RNAs were labelled with [^{3}H]uridine by incubation of ARL cells for 15 min, and irradiated with UV. S1 proteins were prepared as usual by extraction at pH 4.9 from isolated nuclei, which were previously digested extensively with RNase A and DNase I. S1 proteins were cross-linked to [^{3}H]RNAs by UV (Figure 6C, panel II, lane 3); without irradiation, no cross-linking occurred (lane 1). By immunoprecipitation with antibody R2, ^{3}H-labelled protein C1 was almost quantitatively precipitated (lane 4 in Figure 6C, panel II). Protein D1 seemed cross-linked too, but the signal was very weak (lane 4). Under the same conditions, a control antibody, McAb 351, specific for S1 proteins C2 and D2, precipitated these proteins selectively (lane 5). When UV irradiation was done after [^{3}H]uridine-labelling for 2.5 h, cross-linking of C1 to hnRNA was reproducibly observed (Figure 6D, lane 2). Cross-linking of B2 occurred. However, the signal was very weak. These results indicated that the S1 protein C1 was in direct association with hnRNAs in hnRNP complexes. On the other hand, cross-linking of proteins B2 and D1 to hnRNA did not occur significantly.

Structures associated with S1 proteins in hnRNPs

In isolated ARL cell nuclei, hnRNPs were cleaved to 40 S hnRNP particles by an endogenous nuclease(s) [8,39] by incubation for 1.5 h on ice, and the products were analysed by centrifugation on sucrose density gradients (Figures 7A and 7B). 40 S particles, detected with antibody 4E4, which is specific for the particle proteins [40], sedimented with a peak at the sixth or seventh fraction (Figure 7A; a graphic peak position was determined at the '6.4th' fraction by curve-drawing of densitometrically measured core proteins). On the other hand, RNP fragments containing S1 proteins B2 and C1 sedimented as 33 S fragments, with a peak at the eighth or ninth fraction (Figure 7B). [The graphic peak at the '8.4th' fraction and the S value were obtained similarly, using 40 S particles ('6.4th' fraction) as a reference]. In contrast, protein D1 and a fraction of protein C1 were recovered on the top of the gradient. Since D1 and C1 protein-containing intact hnRNPs sedimented with *s* (sedimentation coefficient) values up to 200 S or larger (Figure 6A), this extensive decrease in the sedimentation suggests again that, in hnRNPs, protein D1 and a fraction of protein C1 localized at nuclease-hypersensitive sites.

With rat liver nuclei, the same results as in Figure 7(A) and 7(B) were obtained. Figure 7(C) shows the presence of S1 proteins B2 and C1 in 40 S particle fraction (fraction 6). The S1 proteins (lane 1) and 40 S hnRNP core proteins (lane 3) accounted for 3.2 and 77.7 % of fraction 6 proteins respectively, as determined by densitometry of Coomassie Brilliant Blue-stained bands (lane 5). From these and their molecular masses, S1 proteins and 40 S hnRNP core proteins were estimated to be present in a molar ratio of approx. 1:25 in the fraction 6. In addition, the antibody R2 immunoprecipitated the 40 S hnRNP core proteins more than twice as much as did the preimmune serum, under the conditions employed (Figure 7D, lanes 2 and 3). Essentially the same results were obtained under different conditions (lane 5). The protein molar ratio (1: 25) estimated above suggests that, if distributed evenly, one or two copies of S1 proteins may be found in each

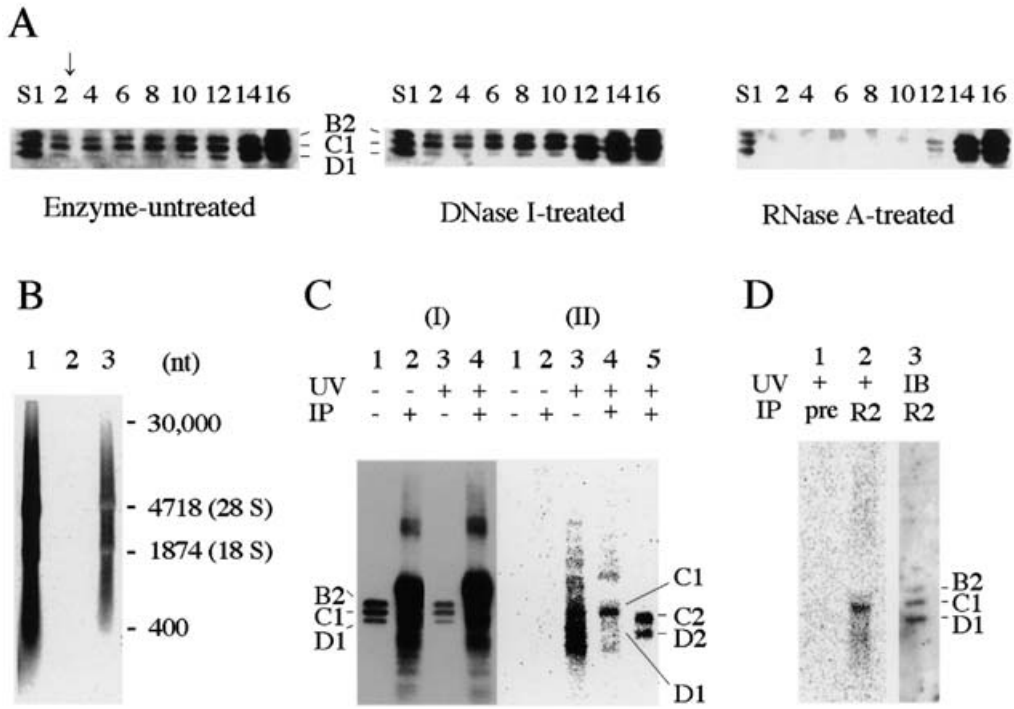


Figure 6 Association of S1 proteins with hnRNPs

(**A**) Sedimentation analysis. Combined second and third extracts from rat HTC-cell nuclei prepared as described in the legend to Figure 5 were incubated with or without 50 μg/ml pancreatic RNase A or DNase I at 20 °C for 15 min and centrifuged on sucrose density gradients. Fractions were collected from the bottom and immunoblotted with antibody R2. Numerals indicate fraction numbers, and the arrow shows the sedimentation position of marker tobacco-mosaic-virus particles (200 S). S1, reference rat liver S1 proteins. (**B**) Association of S1 proteins with hnRNAs. ARL cells were pulse-labelled (15 min) with [^{3}H]uridine in the presence of actinomycin D (at 0.04 μg/ml to decrease rRNA synthesis by about 50 % [42]). Isolated nuclei were sonicated, and the supernatant (lane 1) was immunoprecipitated with a control preimmune serum (lane 2) or antibody R2 (lane 3) bound to Protein A–Sepharose beads. The [^{3}H]RNAs were collected in the presence of unlabelled total RNAs added as a carrier, then electrophoresed. The blot was analysed on an image analyser. The unlabelled RNAs competed with labelled RNAs in the blotting, giving bleached ^{3}H images at 18 S (1874 nt) and 28 S (4718 nt) rRNAs. (**C**) Direct binding of S1 proteins to hnRNAs. ARL cells were labelled with [^{3}H]uridine for 15 min, and [^{3}H]RNAs and bound proteins were cross-linked by UV irradiation. S1 proteins were extracted after extensive digestion of isolated nuclei with DNase I and RNase A. Half of each extract was immunoprecipitated with antibody R2. (I) The samples were immunoblotted with antibody R2; (II) the blot in (I) was analysed for ^{3}H images on an image analyser. Abbreviations: UV, UV-cross-linked, IP, immunoprecipitated. Dense bands on lanes 2 and 4 in (I) are IgGs of polyclonal antibody R2. Lane 5 in (II), proteins immunoprecipitated with a control antibody, McAb 351, that is specific for S1 proteins C2 and D2 [12,43]. (**D**) The same experiment as in (**C**), except for a longer incubation (2.5 h) with [^{3}H]uridine. Lanes 1 and 2, ^{3}H images of proteins immunoprecipitated with a preimmune serum and antibody R2 respectively; lane 3, reference rat liver S1 proteins immunoblotted (IB) with antibody R2.

40 S particle, which contains a total of about 36–48 copies of 40 S hnRNP core proteins, which consist of six molecular species [9].

hnRNP D0 and hnRNP D: their relationship

In two-dimensional gel electrophoresis of rat liver S1 proteins, each protein isoform was resolved into up to six or more spots according to their pI values (Figure 8A). Very similar patterns were also obtained with total extracts of cultured rat ARL cells and hamster HaK cells (results not shown). The multiple spots were due to post-synthetic modifications, one of which was phosphorylation, as suggested by labelling of the spots with [^{32}P]P_i added to the incubation of ARL cells (results not shown). Arginine methylation may also contribute to the multiple spots. From the results some information was derived which is described in detail in the Discussion; namely, the reported spots, not only those of hnRNP D01, hnRNP D02 and hnRNP D1* (an hnRNP having a mobility close to that of hnRNP D1) [24], but also those of hnRNP D1 and D2 [11,30], precisely correspond to the major spots of S1 proteins (Figure 8B). This suggests that the longstanding uncertainty about the relationship between hnRNP D0 and hnRNP D proteins is now understood in terms of splicing and modification isoforms of the same gene products.

Dephosphorylation of S1 proteins with alkaline phosphatase

In the present study, identification of S1/AUF1 isoforms was based primarily on their gel mobility. It was possible that the mobility is altered by modifications, particularly by phosphorylation, which is known to affect often the gel mobility of proteins. ARL cells were incubated with [^{32}P]P_i for 1.5 h, and S1 proteins were analysed. S1 protein D2 was strongly labelled with [^{32}P]P_i. B2, C1 and D1 were also labelled (Figure 9, lane 1), but to a lesser extent. By treatment with alkaline phosphatase, the protein-bound ^{32}P was almost completely removed (lane 2). Noticeably, the phosphate removal increased the electrophoretic mobility of S1 proteins, but only slightly, without alteration of the mobility order of the S1 proteins (lanes 3 and 4). These results thus indicate that gel mobility is a useful index in distinguishing between S1/AUF1 isoforms.

DISCUSSION

The present study showed that S1 proteins B2, C1 and D1 are identical with p45, p40 and p37 AUF1 isoforms respectively. Importantly, they were found largely as hnRNP proteins in the nucleus.

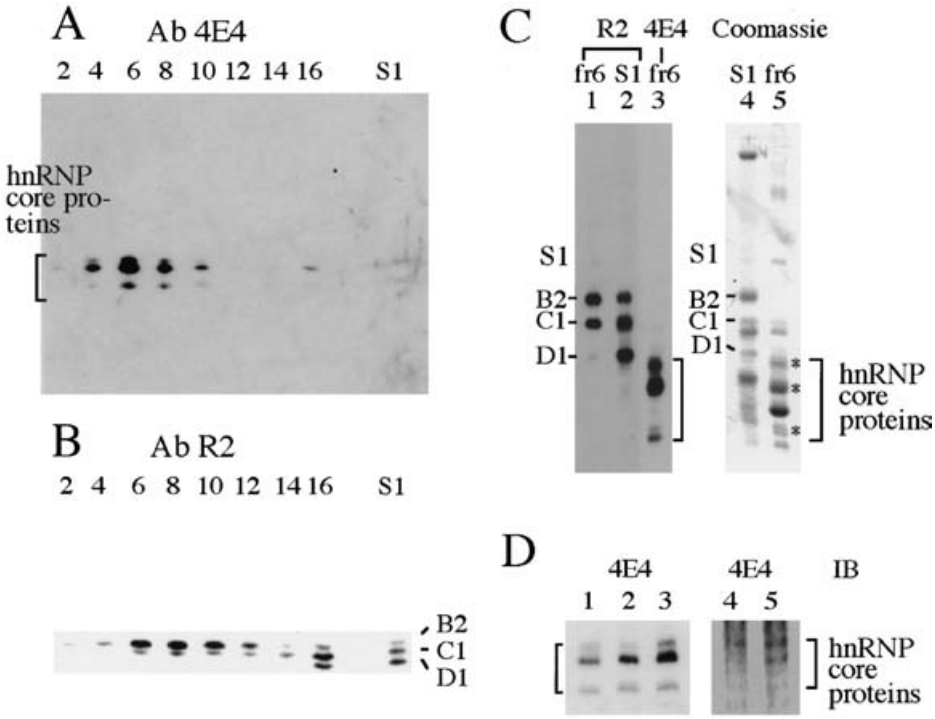


Figure 7 hnRNP substructures associated with S1 proteins

ARL cell nuclei were incubated for a total of 1.5 h at 4 °C, and the combined supernatants were centrifuged on 5–20 % sucrose gradients. Fractions were collected from the bottom and immunoblotted using antibody 4E4, specific for 40 S hnRNP core proteins (**A**), and the blot was re-probed with antibody R2 (**B**). Numerals and square brackets indicate fraction numbers and 4E4 reactive core proteins respectively. S1, reference rat liver S1 proteins. (**C**) A 40 S hnRNP particle fraction was prepared from rat liver nuclei under the same conditions as those described above. The 40 S fraction (fraction 6, 'fr6') and reference S1 proteins ('S1') were immunoblotted using an 18-cm-long SDS-containing gel. The blot was cut longitudinally and stained with antibody R2 (lanes 1 and 2), antibody 4E4 (lane 3) and Coomassie Brilliant Blue (lanes 4 and 5). Asterisks indicate 4E4-reactive 40 S hnRNP core proteins. Six times more proteins were loaded on lanes 4 and 5. (**D**) The 40 S hnRNP particle fraction was immunoprecipitated with a pre-immune serum (175 μg of serum protein) or antibody R2 (125 μg of serum protein) bound to Protein A–Sepharose beads (50 μl packed volume). Precipitated proteins were immunoblotted ('IB') with antibody 4E4. Lanes 1–3, input 40 S fraction (1/10 amount) and the proteins immunoprecipitated with control serum or R2 respectively. In a similar immunoprecipitation (lanes 4 and 5), IgG-bound Protein A–Sepharose beads were previously blocked with 5 % (w/v) non-fat dry milk, and the amount of 40 S fraction used in IP was decreased to one-fifth. Lane 4, a control serum; lane 5, antibody R2.

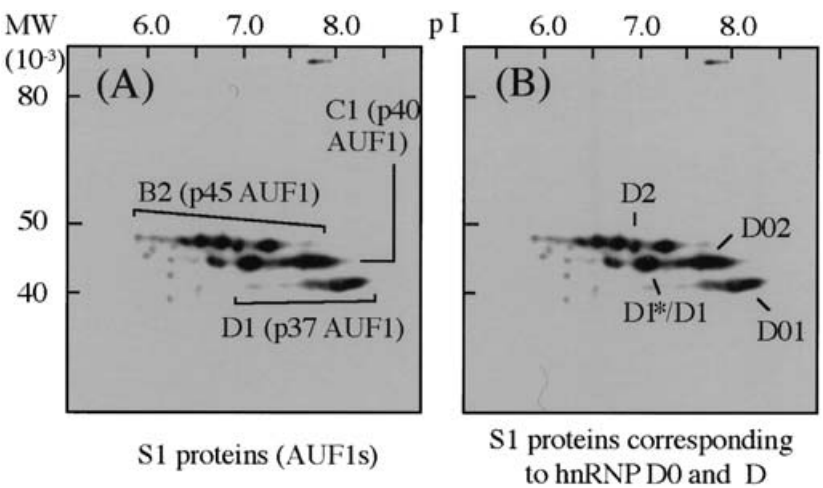


Figure 8 Two-dimensional gel electrophoresis of rat liver S1 proteins

(**A**) Rat liver S1 proteins were prepared by the DNase I digestion method, resolved by two-dimensional gel electrophoresis and immunoblotted with antibody R2. The assignment of S1 protein B2 (p45 AUF1), C1 (p40 AUF1) and D1 (p37 AUF1) were based on their mobility on second-dimensional SDS/PAGE. (**B**) The spots of hnRNP D01, D02, and D1* in [24] and hnRNP D1 and D2 in [11,30], which correspond to those of the S1 proteins in (**A**), are indicated. The assignments were done as described in the Discussion.

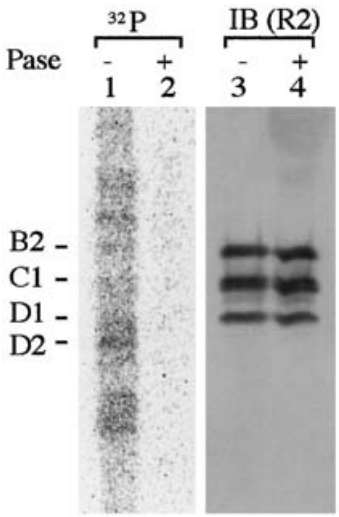


Figure 9 Dephosphorylation of S1 proteins with alkaline phosphatase

ARL cells were incubated with [^{32}P]P_i for 1.5 h, and S1 proteins were extracted at pH 4.9 as usual. The mixture was adjusted to pH 9.1 with Tris base solution and incubated with calf intestinal alkaline phosphatase (Zymed Laboratories, Inc., South San Francisco, CA, U.S.A.; 35 units for the samples from ten 10-cm-diameter plates) in the presence of 0.24 M NaCl, 3 mM $MgCl_2$ and 50 μM $ZnCl_2$. Proteins were resolved by SDS/PAGE using an 18-cm-long gel, and the blot was analysed for ^{32}P image on an image analyser (lanes 1 and 2) and immunostained with antibody R2 (lanes 3 and 4).

S1 proteins were extracted gradually from isolated nuclei with a low-salt solution. In contrast, the bulk of the nuclear proteins was extracted in the first two washings (Figure 5); consequently, the S1 protein concentration in the extracts increased with repeated extraction. Their gradual extraction appears to be consistent with their occurrence as hnRNPs. In isolated nuclei, nascent hnRNPs may be slowly attacked by endogenous nuclease(s) and liberated from the bound RNA polymerases, or transcribed hnRNPs that have been still in progress of processing may leak out gradually through nuclear pores *in vitro*.

By mild nucleolytic cleavage of hnRNPs, protein B2 and a portion of protein C1 were recovered in 33 S RNP fragments. This suggests that, in addition to the basic repeat structure that generates canonical 40 S particles, there is another substructure in hnRNPs that contains S1 proteins and produces 33 S fragments. Interestingly, a small fraction of S1 proteins was found in the 40 S particles. In fact, immunoprecipitation with antibody R2 co-precipitated 40 S core proteins. The precursor–product relationship between 40 and 33 S fragments is an intriguing possibility. However, it is more likely, judging from the distinct sedimentation profiles of 40 S hnRNP core proteins and 33 S particle-constituting S1 proteins (Figures 7A and 7B), that there are compositional variations in the particle-producing hnRNP substructures. hnRNP proteins are generally associated with intron sequences [41]; S1 proteins B2 and C1 (p45 and p40 AUF1s) may be present on introns, forming such hnRNP substructures.

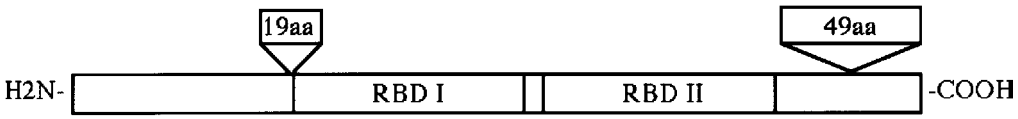
S1 protein D1 and a remaining fraction of protein C1 were present in the nuclease-hypersensitive structures in hnRNPs. Proteins D1 and C1 versus protein B2 occurred in the molar ratio 7.0:0.58 in the cytoplasm, in contrast with that (82:100) in the nucleus (the values represent relative amounts of each protein in liver cells). This indicates that only a small fraction of S1 proteins occurs in the cytoplasm, and the cytoplasmic S1 proteins are mostly D1 and C1 (p37 and p40 AUF1s). Consistently, these AUF1s are exported out from nucleus [23], and involved in the ARE-directed rapid decay of mature mRNAs in the cytoplasm [16]. It is hence suggested that, in hnRNPs, AREs constitute nuclease-hypersensitive structures that contain S1 proteins D1 and C1. D1 was present at significantly low levels in all examined cell types (Figures 1 and 3C). Probably its small cellular amount, as compared with other S1 proteins, is sufficient for its essential role for the cytoplasmic function.

UV cross-linking showed that S1 protein C1 (p40 AUF1) was directly bound to hnRNA *in vivo*. Protein B2 (p45 AUF1) seems to have been cross-linked too, but to a much lesser extent than would be expected in view of its abundance. Cross-linking of protein D1 (p37 AUF1) to hnRNAs was not significant either. The binding of B2 and D1 to hnRNA may occur indirectly, by the formation of oligomers with protein C1 or other RNA-binding protein [19].

hnRNP D0 is identical with AUF1 [27,29]. In spite of the name it has been given, hnRNP D0 has not been characterized as an hnRNP protein. The present study verified that hnRNP D0 (AUF1) proteins are indeed hnRNP proteins. The present results further suggested that hnRNP D0 and hnRNP D are splicing isoforms and charge-modification isoforms arising from one gene. This notion was arrived at as follows: (1) S1 proteins B2, C1 and D1 were identical with p45, p40 and p37 AUF1 proteins; hence S1 proteins = AUF1 isoforms = hnRNP D0s; (2) hnRNP D0 proteins had been so named because of their reactivity to an anti-(hnRNP D) monoclonal antibody and their migration, in two-dimensional gel electrophoresis, near to, but separate from, hnRNP D proteins (DO1, D1* and D02 versus D1 and D2). Molecular sizes are in the order of D01 < D02 = D1* = D1 < D2 [24]; and (3) the two-dimensional spots of hnRNP D0s shown in Figure 6(A) and 6(B) in [24], and those of hnRNP Ds in Figure 3 in [30] and Figure 1 in [11] correspond perfectly to the major spots of S1 proteins B2, C1 and D1 (Figure 8B). Thus it is suggested that the longstanding uncertainty in the relationship between hnRNP D and hnRNP D0 is due to the presence of multiple charge isoforms and splicing isoforms produced from the same gene. Their relationship is summarized in Figure 10.

The previous discordance of hnRNP D0 and hnRNP D seems to have arisen from different characterization methods. hnRNP D1 and hnRNP D2 were isolated as hnRNP proteins by co-immunoprecipitation with an antibody against a 40 S hnRNP core protein, from the extracts of HeLa cells metabolically labelled with [^{35}S]methionine [30]. That hnRNP D1 and D2 proteins (S1 proteins C1 and B2) occur mainly as hnRNP proteins among the isoform proteins was confirmed in the present study. It is thought that in the previous study [30], the hnRNP D1 and D2 spots have been detected on two-dimensional gels owing to their higher abundance and high incorporation of radioactivity. In fact, more charge isoforms of these proteins were later detected after a long film exposure (Figure 1 in [11]). On the other hand, hnRNP D0 proteins were isolated by extensive purification [24], in which the isoform corresponding to hnRNP D2 seems to have been lost at some purification step(s).

In the present study, the antibody R2 detected all modified forms, as well as all splicing isoforms (Figure 3A) of S1 proteins, as judged from perfect overlapping of Coomassie Brilliant Blue- and immuno-stained spots on a two-dimensional blot of rat liver S1 proteins (results not shown). Therefore it is highly likely that the present conditions allow all the AFU1/hnRNP D0/hnRNP D proteins to be observed, and reveal also that S1 proteins undergo extensive modifications. The modification reactions responsible for different charge isoforms may be important for controlling the diverse cellular functions of S1/AUF1/hnRNP D0/hnRNO D proteins, which range from the formation of hnRNP structures to the cytoplasmic mRNA turnover, as well as transcriptional regulation and telomere maintenance.



Name	(19-aa insert/ 49-aa insert)				Designated in	Ref.
	(+/+)	(-/+)	(+/-)	(-/-)		
(aa)	354	337	304	285		
S1 protein	B2	n	C1	D1	Selective extraction	[1]
AUF1	p45	p42	p40	p37	cDNA cloning	[27]
hnRNP D	D2	n	D1	n	2-D gel e. f.	[30]
hnRNP D0	n	n	D02, D1*	D01	2-D gel e. f.	[24]
	+/+	-/+	+/-	n	cDNA cloning	[26]
	D0D	n	D0B	n	cDNA cloning	[20]

Figure 10 Relationship of S1 proteins with isoforms of AUF1, hnRNP D0 and hnRNP D

hnRNP D0 proteins (AUF 1 proteins) contain two non-identical RNA-binding domains (RBDI and RBDII) [26]. Owing to alternative splicings, there are four isoforms with or without N-terminal 19- and C-terminal 49-amino-acid inserts. Sequences of AUF1 cDNAs [27] have been shown to be identical with those of three cDNA variants (+/+, −/+ and +/−) of hnRNP D0s [26]. The smallest variant without inserts (p37 AUF1) was also cloned in [27]. These isoforms, and their spots on two-dimensional gel electrophoresis have been called by various names. hnRNP D0B and D0D are those reported by Tolnay et al. [20]. aa indicates the number of amino acid residues of rat AUF1 proteins (GenBank® accession numbers ABO46615–ABO46618).

To sum up, the present results demonstrate that S1 proteins B2, C1 and D1 are identified with AUF1 proteins and exist mostly as hnRNP proteins in the cell nucleus. That hnRNP D0 is indeed an hnRNP protein was verified.

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