A Novel Heterogeneous Nuclear RNP Protein with A Unique Distribution on Nascent Transcripts

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Abstract. Immediately after the initiation of transcription in eukaryotes, nascent RNA polymerase II transcripts are bound by nuclear proteins resulting in the formation of heterogeneous nuclear ribonucleoprotein (hnRNP) complexes. hnRNP complexes from HeLa cell nuclei contain >20 major proteins in the molecular mass range of 34,000–120,000 D. Among these are the previously described A, B, and C groups of proteins (34,000–43,000 D) and several larger, and as yet uncharacterized, proteins. Here we describe the isolation and characterization of a novel hnRNP protein termed the L protein (64–68 kD by mobility in SDS–polyacrylamide gels). Although L is a bona fide component of hnRNP complexes, it also appears to be a different type of hnRNP protein from those previously characterized. A considerable amount of L is found outside hnRNP complexes, and monoclonal antibodies to the L protein also strongly stain unidentified discrete nonnucleolar structures, in addition to nucleoplasm, in HeLa cell nuclei. Interestingly, the same antibodies stain the majority of nonnucleolar nascent transcripts from the loops of lampbrush chromosomes in the newt, but the most intense staining is localized to the landmark giant loops. The L protein is the first protein of giant loops identified so far, and antibodies to it thus provide a useful tool with which to study these unique RNAs. In addition, isolation and sequencing of cDNA clones for the L protein from human cells predicts a glycine- and proline-rich protein of 60,187 D, which contains two 80 amino acid segments only distantly related to the RNP consensus sequence-type RNA-binding domain. The L protein, therefore, is a new type of hnRNP protein.

1. Abbreviations used in this paper: CS-RBD, ribonucleoprotein consensus type RNA binding domain; GV, germinal vesicle; hnRNP, heterogeneous nuclear ribonucleoprotein; RNP-CS, ribonucleoprotein consensus sequence; ss, single stranded.
vivo (Mayrand et al., 1981; Dreyfuss et al., 1984b) indicating they are bound to hnRNA in the cell.

To facilitate the production of monoclonal antibodies to these novel hnRNP proteins, we have used the ability of most hnRNP proteins to bind single-stranded (ss)DNA in a heparin- and salt-resistant manner (Pandolfi et al., 1987; Piñol-Roma et al., 1988). Here we characterize one of these abundant higher molecular mass hnRNP proteins, the L protein. In comparison to other hnRNP proteins characterized so far, L is unique in its distribution on nascent transcripts and in its amino acid sequence. Of particular interest is the finding of an association of L with a distinct set of nascent transcripts—those of the landmark giant loops of amphibian lambrush chromosomes. L is the first protein found to localize to these nuclear structures, and it is likely that the antibodies to L that have been produced will make it possible to learn more about the giant loops.

Materials and Methods

Cell Culture, Labeling, and Cell Fractionation

HeLa S3 and the HeLa monolayer-adapted clone JW36 cells were cultured in monolayer to subconfluent densities in DME, supplemented with penicillin and streptomycin, and containing 10% calf serum at 37°C. Cells were labeled with [35S]methionine at 20 μCi/ml for 20 h in DME containing one-tenth the normal methionine level and 5% calf serum. The nucleoplasmic fraction was prepared essentially according to Pederson (1974), as previously detailed (Choi and Dreyfuss, 1984a).

RNase Digestion

Digestions of the nucleoplasmic fraction were carried out with micrococcal RNase I bound to protein A-agarose. Rabbit anti-mouse IgG antiserum was PifioI-Roma et al., 1988). The anti-L protein monoclonal antibody 4DII and the anti-A1 monoclonal antibody 4B10 (Piñol-Roma et al., 1988). Detection of the mouse antibodies was with fluorescein isothiocyanate–conjugated goat anti-mouse IgG (1:200) as the secondary antibody.

Preparation of Monoclonal Antibodies

The monoclonal antibodies 4F4, to the C proteins, and 4B10, to the A1 protein, were prepared as described previously (Choi and Dreyfuss, 1984a; Piñol-Roma et al., 1988). The anti-L protein monoclonal antibody 4DII was obtained by immunization of a BALB/c mouse with hnRNP proteins purified by affinity chromatography on ssDNA agarose (Piñol-Roma et al., 1988). The antigen was composed of proteins eluting from the column at 2 M NaCl after a heparin wash at 1 mg/ml in 100 mM NaCl. Hybridoma production and screening were as previously detailed (Choi and Dreyfuss, 1984a).

Immunopurification of Proteins and hnRNP Complexes

The hnRNP complex was immunopurified from the nucleoplasm as described previously (Choi and Dreyfuss, 1984a) for 10 min at 4°C with the anti-C proteins monoclonal antibody 4F4 or anti-L monoclonal antibody 4DII bound to protein A–agarose. Rabbit anti–mouse IgG antiserum was used with the 4DII antibody, since 4DII does not bind protein A directly. The same secondary antiserum was included with all the SP2/0 nonimmune controls. Ascites fluid from a BALB/c mouse that was inoculated intraperitoneally with the parent myeloma line SP2/0 was used for the nonimmune control immunopurifications with each experiment. Antibody specificities were confirmed by immunoblotting and by immunopurification in the presence of theionic detergent Empigen BB at 1%, 0.1 mM EDTA, and 0.1 mM DTT as described (Choi and Dreyfuss, 1984b).

Gel Electrophoresis and Immunoblotting

Protein samples were subjected to electrophoresis on an SDS-containing discontinuous PAGE system (SDS-PAGE) (Dreyfuss et al., 1984a). The separating gel had a final acrylamide concentration of 12.5%. After electrophoresis of [35S]methionine-labeled proteins, the gel was stained with Coomassie Blue and impregnated with 2,5-diphenyloxazole for fluorography (Laskey and Mills, 1975). Two-dimensional NEPHGE was carried out by the procedure of O'Farrell et al. (1977). The first dimension was separated by using pH 3–10 ampholine gradients for 4 h at 400 V, and the second dimension was by SDS-PAGE as described above. Immunoblotting procedures were as described previously (Choi and Dreyfuss, 1984b).

Sucrose Gradient Sedimentation

Sucrose gradients (10–30% [wt/vol]) were sedimented in a rotor (model SW41; Beckman Instruments Inc., Palo Alto, CA) at 38,000 rpm for 5 h at 4°C; 22 fractions (0.6 ml each) were collected from the bottom, and proteins in each fraction were precipitated with trichloroacetic acid added to a final concentration of 10% for analysis by SDS-PAGE and immunoblotting as described above. Sucrose solutions were made up of 10 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl and 2.5 mM MgCl₂. The 28S ribosomal marker was from phenol-extracted cytoplasmic fraction sedimented similarly in the presence of 10 mM EDTA.

Immunofluorescence Microscopy of Human Cells

Immunofluorescence microscopy was essentially as previously described (Dreyfuss et al., 1984b). Monolayer-adapted HeLa cells (clone JW36), cultured on glass coverslips, were fixed with 2% formaldehyde in PBS for 30 min at room temperature, followed by permeabilization with acetone at −20°C for 3 min. Ascites fluid dilutions were 1:1,000 for both 4DII and the anti-A1 monoclonal antibody 4B10 (Piñol-Roma et al., 1988). Detection of the mouse antibodies was with fluorescein isothiocyanate–conjugated goat anti-mouse IgG (1:200) as the secondary antibody.

RNA-Protein Cross-linking in Intact Cells

Photochemical RNA-protein cross-linking by UV light irradiation of cells on culture dishes and isolation and analysis of RNPs was carried out as previously described (Dreyfuss et al., 1984a,b).

Isolation of cDNA Clones and Affinity Purification of Antibodies

Mouse antisera (1:250 dilution) were used to directly screen a Agt11 HeLa cell cDNA library (Nagakawa et al., 1986). Positive plaques were purified, plated at high density (5 × 10⁵ phage/100-mm plate), and filter replicated onto 82-mm nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH). These filters were used to affinity purify antibodies from the total mouse antisera using the technique of Snyder et al. (1987). These antibodies affinity selected by the filter-bound fusion proteins were then used to screen Western blots of total HeLa cell proteins to identify L protein clones. A full-length clone, pHCL3, was subsequently isolated using the original L protein clone, pHCL1, as a hybridization probe.

RNA Blot Analysis, Hybrid Selection, and In Vitro Translation

Poly(A)+ RNA was prepared from HeLa S3 cells as previously described (Nagakawa et al., 1986), resolved by electrophoresis on formaldehyde-containing 1.4% agarose gels (Lehrach et al., 1977), and the fractionated RNA blotted onto nitrocellulose (Maniatis et al., 1982). Both pHCL1 and pHCL3, and various subfragments, were used as hybridization probes, and were prepared by nick translation with [32P]dCTP (Rigby et al., 1977). Hybridization selection and in vitro translations were performed as previously described (Nagakawa et al., 1986; Swanson et al., 1987) using either pHCL2, a clone for the human C proteins, or pHCL1.
DNA Sequence Analysis

Overlapping restriction fragments of pHCL1 and pHCL3 were subcloned into M13mpl8 and M13mpl9 and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using deaza-GTP and the Klenow fragment of DNA Polymerase I as described previously (Swanson et al., 1987). Sequence analysis was performed with the University of Wisconsin Genetics Computer Group (UWCG) Sequence Analysis Programs. The universal sequence database searching programs FASTA and TFASTA (Pearson and Lipman, 1988) were used to search six databases for sequence similarities.

Results

L Protein Is a Component of Immunopurified hnRNP Complexes

The protein composition of hnRNP complexes isolated from nucleoplasm of human HeLa cells with monoclonal antibodies to the hnRNP A1 or C proteins has been described previously (Choi and Dreyfuss, 1984a; Piñol-Roma et al., 1988). These complexes are composed of ~20 major proteins in the molecular mass range of 34,000-120,000 D as resolved by two-dimensional gel electrophoresis, and these are designated alphabetically as shown in Fig. 1 left (see Piñol-Roma et al., 1988 for detailed discussion). A major component of hnRNP complexes obtained by immunopurification is a group of proteins of ~64-68 kD, referred to as L and M. Proteins of similar molecular mass are some of the major proteins that become cross-linked to RNA by UV light in vivo (Dreyfuss et al., 1984b), and therefore it was of interest to produce specific probes for these proteins to facilitate their study. Antibodies to the L protein were generated by immunizing mice with fractions containing HeLa hnRNP proteins partially purified by affinity chromatography on ssDNA-agarose, a procedure that enables the large scale purification of most hnRNP proteins (Piñol-Roma et al., 1988). Reactivity towards several of these proteins was observed, and a mouse that showed good response against the L protein was used for production of monoclonal antibodies. The specificity of the monoclonal antibody produced by one of the stable hybridomas, 4D11, for the L protein is shown by two-dimensional gel electrophoresis of material immunoprecipitated from total HeLa cell proteins in the presence of the ionic detergent Empigen BB (Choi and Dreyfuss, 1984b) (Fig. 1 right). A comparison of the proteins immunopurified in Fig. 1 right and left also demonstrates that 4D11 only recognizes a subset of the proteins in the region of L and M, hence the distinction made among proteins in this region. Several isoelectric forms, probably the result of posttranslational modifications, are seen for both L and M. A series of monoclonal antibodies specific for M (Adam, S., S. Piñol-Roma, and G. Dreyfuss, unpublished data), as well as polyclonal antisera against L, confirm the immunological relatedness of the proteins within each group, and the lack of immunological cross-reactivity between the two groups.

Further evidence that L is an authentic component of hnRNP complexes was obtained by using 4D11 in immunopurification experiments starting with HeLa nucleoplasm with or without addition of ionic detergent. In the presence of

Figure 1. Two-dimensional gel electrophoresis of immunopurified hnRNP complexes and immunopurified L protein. (Left) hnRNP complexes were immunopurified from [35S]methionine-labeled HeLa nucleoplasm with the anti-C proteins monoclonal antibody 4F4 as described in the text. The immunopurified complexes were resolved by two-dimensional gel electrophoresis, with NEPHGE in the first dimension, and SDS-PAGE in the second dimension. The proteins were visualized by autoradiography. (Right) L protein was immunopurified from [35S]methionine-labeled HeLa cells with the 4D11 monoclonal antibody, in the presence of the ionic detergent Empigen BB in order to dissociate protein–RNA and protein–protein interactions. The immunopurified protein was then resolved by two-dimensional gel electrophoresis simultaneously with the sample shown on the left to allow for direct comparison of electrophoretic behavior of the individual proteins.

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L Protein Is Cross-linked by UV Light to Poly(A)-containing RNA in Intact Cells

To examine whether L is indeed in contact with hnRNA in the living cell, we induced covalent cross-linking of RNA to proteins that are associated with it in intact cells by irradiation with UV light. This procedure allows selection of RNA with the covalently bound proteins, under conditions that eliminate adventitious association of proteins with the RNA during the fractionation procedures (van Eekelen et al., 1981; Mayrand et al., 1981; Dreyfuss et al., 1984a,b). HeLa cells grown in monolayer culture were irradiated with UV light, and poly(A)-containing RNA was isolated by affinity chromatography on oligo(dT)-cellulose, after boiling with SDS and β-mercaptoethanol. The proteins that are cross-linked to the selected RNA were released by digestion of the RNA with ribonucleases, and resolved by electrophoresis on SDS-containing polyacrylamide gels. An immunoblot of such a gel probed with the anti-L antibody 4D11 is shown in Fig. 3. It demonstrates that the L protein is bound to...
Figure 4. Sucrose gradient sedimentation behavior of the L protein and of the C proteins. Nucleoplasm from HeLa cells was sedimented on 10-30% sucrose gradients as described in the text, after digestion with micrococcal nuclease at 0 (-MN) or 2 (+MN) U/ml. 600-µl fractions were collected from the bottom. The proteins in each fraction were precipitated with trichloroacetic acid, and the distribution of the L and C proteins was analyzed by SDS-PAGE and immunoblotted with the monoclonal antibodies 4D11 and 4F4 on the same gels. Lane 1 corresponds to the bottom fraction.

Poly(A)-containing RNA in the living cell, and thus further supports the conclusion that it is an authentic hnRNP protein. The mobility shift of L in the UV(+) lane (Fig. 3), as well as the diffuse nature of the signal obtained, are characteristic of proteins that have become cross-linked to RNA, and are the result of residual nucleotides that remain covalently bound to the protein even after exhaustive nuclease digestion. The specificity of the cross-linking is further substantiated by the fact that no 4D11-reactive material is detected if the cells are not exposed to UV light before the RNA isolation procedure (Fig. 3, UV--). The lower molecular mass bands seen in the total lane in Fig. 3 are most likely proteolysis products of L since their presence is variable between preparations.

L Protein Is Associated with RNase-sensitive Structures

Given the indications that considerable amounts of L may be found outside of hnRNP complexes that can be immunopurified with antibodies to the C proteins (Fig. 2), the association of the L protein with RNA-containing structures and its relationship to other hnRNP proteins was examined by sucrose gradient sedimentation. The association of hnRNA with hnRNP proteins results in the sedimentation of hnRNP complexes in a heterodisperse manner on sucrose gradients (Samarina et al., 1968). Mild digestion of the RNA by endogenous nucleases or by added RNases results in a shift of the labeled RNA and associated proteins entering the gradient.
4D11 (see Fig. 1) and 4F4. As shown in the corresponding immunoblots in Fig. 4 (-MN), the L protein sediments throughout the gradient if nuclease treatment of the nucleoplasm is omitted before sedimentation. There appears to be a considerable amount of the protein sedimenting near the top of the gradient, in addition to L protein in hnRNP complexes, that is either free or is part of other structures. The association of the L protein with RNA-containing structures is indicated by the shift in their sedimentation towards the top of the gradient after mild RNAse digestion of the nucleoplasm (Fig. 4, +MN). There also appears to be a strong bias towards the preferential appearance of a minor band of L, with a higher apparent molecular mass, towards the bottom of the gradient. This higher molecular mass form of L is also apparent by immunopurification with 4D11 (see Fig. 1 right and Fig. 2). The relationship between these two forms of the protein is at present unclear, but it appears that they may associate differentially with RNA-containing complexes, with the higher molecular mass band exhibiting a preferential association with faster sedimenting structures. As explained in the previous section, the lower molecular mass bands that react with mAb 4D11, and which are especially prominent in the +MN panel, are most likely proteolytic fragments of L. As a reference, the sedimentation pattern of the well-characterized hnRNP C proteins is shown by immunodetection with the mAb 4F4 on the same blots.

**L Protein Is Conserved in Vertebrate Cells**

Because L is a novel hnRNP protein, we wanted to ascertain that it is a general component of hnRNP complexes rather than a protein unique to HeLa cells. Immunoblotting of material from a variety of vertebrate cells using the anti-L monoclonal antibody 4D11 (Fig. 5) indicates that the L protein is found in various vertebrates including *Xenopus laevis* (data not shown) and the newt *Notophthalmus viridescens*. No detectable signal was found in cells of *Drosophila melanogaster* or in the yeast *Saccharomyces cerevisiae* (data not shown). The apparent molecular mass of the signal obtained with 4D11 across such a wide range of organisms is also remarkably conserved. The high degree of cross-reactivity of the 4D11 antibody across vertebrate species makes it possible to carry out studies in other organisms that would otherwise be difficult to perform in human cells, such as those described below using the lampbrush chromosomes of *N. viridescens*.

**L Protein Is Found in Nucleoplasm and in Unidentified Nuclear Structures**

Immunofluorescence microscopy on human JW36 cells with the mAb 4D11 demonstrates the nuclear localization of these proteins and their absence from the cytoplasm (Fig. 6 B). The pattern of staining is similar to that obtained with monoclonal antibodies against other hnRNP proteins, such as with the anti-A1 protein mAb 4B10 (see Fig. 6 A), in that the overall staining is nucleoplasmic with the exclusion of nucleoli. However, 4D11 also strongly stains one to three discrete (usually two) nonnucleolar structures that are apparent in all cells. The precise identity of these structures is at present unknown, but they are not observed with anti-C, anti-A1, and anti-U antibodies which show only nucleoplasmic staining. Identical patterns of staining have also been obtained with additional monoclonal antibodies against L, as well as with polyclonal antisera raised against an L–β galactosidase fusion protein (data not shown). We have also observed similar staining in mouse tissue culture cells and tissue sections.

**Distribution of the L Protein on Nascent Transcripts**

To examine the intranuclear distribution of L in greater detail, we stained lampbrush chromosome preparations of the newt *N. viridescens* with mAb 4D11. The great majority of the lateral loops bound the antibody, suggesting that L is associated with most nascent transcripts on the chromosomes. Binding of 4D11 to the cluster of giant loops near the centromere of chromosome 2 was especially striking (Fig. 7, A and B). Even when allowance is made for the greater thickness of the loop matrix on the giant loops, it seems probable that the concentration of L is higher in them than in typical loops. The staining of the giant loops by 4D11 is unusual in another respect. Other antibodies that stain typical lampbrush chromosomes stain the giant loops only faintly or not at all (Roth and Gall, 1987). For example, mAb Y12, which is directed against the Sm epitope of snRNPs (Lerner et al., 1981), stains most lampbrush chromosome loops, but leaves the giant loops only slightly above background level (Fig. 7, C and D).
In the intact germinal vesicle (GV) the giant loops on chromosome 2 are surrounded by a cloud of small, irregularly shaped granules. During centrifugation of the lampbrush chromosomes for cytological analysis, these granules come to lie in the general vicinity of the giant loops. They also stain intensely with mAb 4D11 (Fig. 7, A and B), suggesting that products from the giant loops are being shed regularly into the nucleoplasm. The hundreds of extrachromosomal nucleoli and a variety of other particulates in the nucleoplasm fail to stain with 4D11. Sections of immature newt ovary were fixed by freeze-substitution, embedded in paraffin, and sectioned at 4 μm. After staining with mAb 4D11, the germinal vesicle contents were more or less uniformly stained except for the nucleoli. In some oocytes an intensely fluorescent mass within the GV was easily recognizable above the general level of staining (not shown). We presume that this mass contains the giant loops.

Isolation of cDNA Clones for the L Protein

Coincident with the isolation of the mAb 4D11 monoclonal antibody, the mouse antiserum was used for isolation of cDNA clones. Fig. 8 (lane total) shows that the serum of one immunized mouse reacted with several hnRNP proteins in-
Figure 7. Immunofluorescence microscopy with monoclonal antibodies 4D11 and Y12 on newt lampbrush chromosomes. (A and B) Antibody 4D11. Portion of lampbrush chromosome 2 from the newt Notophthalmus viridescens showing the giant loops near the centromere. (A) Phase-contrast and (B) fluorescence images of the same region after staining with 4D11 and rhodamine-labeled second antibody. The giant loops are intensely stained, as are numerous extrachromosomal granules that regularly accompany these loops. The majority of typical loops are also stained by the antibody, but less intensely. (C and D) Antibody Y12. (C) Phase-contrast and (D) fluorescence images of the same region of chromosome 2 after staining with mAb Y12, which is directed against the Sm epitope of snRNP proteins. Most typical loops are well stained, but the giant loops are barely detectable. Y12 also stains numerous small, spherical granules in the nucleoplasm, but not the irregular granules that accompany the giant loops (note the unstained patches to the left of the giant loops). Bar, 50 μm.
RNA of ~2.3 kb. Using the hcL1 clone as a hybridization probe, a cDNA clone containing the entire protein coding region was isolated, phcL3 (Fig. 11 A). This clone codes for the entire L protein by two different criteria. (a) In vitro transcription and translation of pHCL3 yields a protein that comigrates with the hybrid selection/translation product shown in Fig. 9 and with authentic L protein isolated from HeLa cells by immunopurification with the mAb 4D11. (b) Other L protein cDNA clones that contain sequence information upstream of the 5' end of the cDNA reported in Fig. 11 B contain stop codons in all three reading frames up-

cluding A1, A2, C1, C2, L, and U and an unidentified protein of ~90 kD. This serum was used to directly screen a HeLa cell Agt11 cDNA library, and positive plaques were selected and purified. As previously described for the isolation of the yeast mRNA poly(A)-binding protein (Adam et al., 1986), these purified phage were used to epitope-select and purify antibodies directed against specific proteins. As also illustrated in Fig. 8 (lane epitope-selected) one of these clones, hcL1, selected antibodies that specifically recognized the hnRNP L protein. Subsequently, the mAb 4D11 was shown to also recognize the fusion protein produced by this phage (data not shown). To further demonstrate that the hcL1 clone encoded the L protein, this clone was used to hybrid-select mRNA from total poly(A)+ RNA, and the specifically selected RNA was translated in vitro. As Fig. 9 shows, the hcL1 clone hybrid-selected mRNA which translated into an ~68-kD protein (panel total, lane hcL1). This protein was specifically immunopurified with 4D11 (Fig. 9, panel immunopurified, lane hcL1) and it was not found in the nitrocellulose control without DNA (lanes C in both panels). As a positive control, and for comparison purposes, a clone for the hnRNP C proteins (Swanson et al., 1987) was used to hybrid-select mRNA for the C1 and C2 proteins (Fig. 9, lanes hcL2).

**Figure 9.** Hybrid selection and in vitro translation using phcL1. HeLa poly(A)+ RNA was hybridized to filter-bound DNAs, eluted, and translated in vitro in a rabbit reticulocyte lysate in the presence of [35S]methionine. The filter-bound DNAs were either a cDNA clone for the hnRNP C proteins (lanes hcL2), or the expression clone for the L proteins (lanes phcL5), or a control (lanes C) in which no DNA was bound to the filter. The proteins produced from these poly(A)+ RNAs by in vitro translation were either directly fractionated by SDS-PAGE (lanes total) or first immunopurified (lanes immunopurified) with the anti-C protein monoclonal antibody 4F4 (lane immunopurified, hcL2) or the anti-L protein monoclonal 4D11 (lane immunopurified, hcL1).

**Figure 8.** Immunoblots using total and affinity-selected antisera. Total HeLa cell proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, and probed with either total mouse antisera raised against a 2 M NaCl fraction from ssDNA agarose (lane total) or antibodies selected by immobilized fusion proteins (lane epitope-selected) expressed by the purified Agt11 clone, phcL1.
Reduced either by proteolysis (Herrick and Alberts, 1976; Buetler et al., 1987; Dreyfuss et al., 1988). The RNP-CS type RNA binding domain (CS-RBD) is a true RNA binding domain since protein segments including primarily this region, proteins (20% amino acid identity when optimally aligned in Fig. 11 C) which are weakly related to each other and to the CS-RBDs of the other hnRNP and snRNP proteins (20% amino acid identity when optimally aligned in Fig. 11 C). As shown in Fig. 11 C, the most highly conserved segment of this weakly repeating region is an octapeptide, which is intriguingly similar to the RNP-CS octapeptides found in the snRNP U1A and U2B' proteins (RGQAFVIF in domain 1 and HDIAFVEF in domain 2 of both proteins) (Sillekens et al., 1987). One of the most highly conserved positions in the entire 90 amino acid CS-RBD is the fifth position of the RNP-CS octapeptide (underlined above in the U1A and U2B' sequences) that is usually a phenylalanine which in the case of A1 has been shown to readily cross-link to DNA oligonucleotides (Merrill et al., 1988). In L, this position is a leucine or methionine which clearly distinguishes this protein from the normal RNP-CS although other amino acids have been found in this position, as is the case for the first domain of the Drosophila sex-lethal protein that contains a serine residue at this position (Bandziulis et al., 1989).

Computer searches of the protein data banks did not reveal significant similarities with any known proteins although some sequence similarity was detectable between the amino terminus of the L protein and the carboxy domain of the hnRNP A1 protein (22.5% identity in an 89 amino acid overlap), and a variety of proline-rich and other glycine-rich proteins also shared a limited degree of sequence similarity. The predicted secondary structure of the L protein, obtained using the Chou-Fas and PlotChou of the UWGCG programs, suggests a protein that contains a small number of short alpha-helical domains and several long stretches of predicted large hydrophobic moment.

**Discussion**

We describe here a novel constituent of hnRNP complexes, the L protein. L is the first non-A, -B, -C type hnRNP protein that has been extensively characterized. The L protein is a bona fide hnRNP protein, and it is a constituent of the same hnRNP complexes that can be immunopurified with antibodies to the A1 and C proteins. Its abundance in immunopurified hnRNP complexes is comparable to that of the B1, B2, and C2 proteins (Piñol-Roma et al., 1988). Along with other hnRNP and snRNP proteins, it is localized on the majority of lampbrush chromosome loops of the newt Notophthalmus viridescens and is, therefore, probably associated with most nascent transcripts. However, L exhibits several properties that set it apart from other hnRNP proteins for which immunological probes and sequence data are available, indicating that it represents a new type of hnRNP protein. Among the unique characteristics of L is its occurrence also outside of the previously defined hnRNP complex. This is evident from the immunopurification experiments and from the sedimentation profiles of L in sucrose gradients, but it is most vividly apparent from immunofluorescent microscopy on somatic nuclei and spread amphibian lampbrush chromosomes. The distinct distribution of L by all these criteria contrasts with that observed for other hnRNP proteins such as A1, C, and U.

The analysis of the distribution of the L protein on lampbrush chromosomes is particularly instructive. Among the antibodies that stain lampbrush chromosomes, 4D11 is the only one that stains typical loops and the giant loops on chromosome 2. In Fig. 7 B the typical loops appear to be poorly stained, but this figure was purposely underexposed in order not to wash out detail in the brilliantly fluorescent giant loops. In fact, 4D11 stains typical loops about as brightly as...
Figure II. Structure of the L protein. (A) Restriction map of phcL1 and phcL3. The protein coding region of the cDNAs is indicated above the restriction maps of the expression clone, phcL1, and the full-length clone, phcL3, as a black box. The polyadenylation signals at the 3' end of the phcL3 cDNA are also indicated above the restriction maps of the expression clone, phcL1, and the full-length clone, phcL3, as a black box. The nucleotide and deduced amino acid sequence of the L protein. Both the phcL1 and phcL3 clones were completely sequenced on both strands using overlapping restriction fragments subcloned into M13 vectors. The only open reading frame sufficiently large to encode the L protein is shown below the nucleotide sequence. (C) Sequence alignment of the two ~80 amino acid regions within the L protein which share a limited degree of sequence similarity to each other and to the RNP-CS RNA-binding domain (~20%). Identical amino acids are indicated with an asterisk, and the more highly conserved octapeptide is marked by a stippled box.
Y12, shown in Fig. 7D. All other antibodies that stain typical loops, including some against known hnRNP proteins, stain the giant loops very weakly or not at all (Roth and Gall, 1987; unpublished observations). Antibodies that show this pattern include mAb iD2 against A and B proteins (Leser et al., 1984), mAb 3G6 against the U protein (Dreyfuss et al., 1984b), and mAbs SE5 and UA5 against newt GV proteins (Roth and Gall, 1987). The anti-snRNP antibody Y12 (Lerner et al., 1981) shows the same pattern of loop staining, but also stains the structures known as "spheres" and numerous smaller nucleoplasmic granules (Fig. 7, C and D). It is abundantly clear, therefore, that the giant loops are deficient in a set of common hnRNP and snRNP proteins found on typical loops. The giant loops also contain unique associated antigens not present at detectable levels in the typical loops. This is shown by their staining with two mAbs that do not stain typical loops, mAb A1 (Lacroix et al., 1985) and mAb TH2 (Roth and Gall, 1987). Thus, the transcripts from the giant loops are associated with the L protein and at least two other antigens, perhaps in the form of specialized hnRNP complexes.

Only limited information is available concerning transcription on the giant loops. Transcription on them, like that on typical loops, is inhibited by α-amanitin at 0.5 μg/ml, and is presumably carried out by RNA polymerase II (Schultz et al., 1981). The efficiency of incorporation of the four ribonucleotides is rather different from that seen in typical loops, suggesting that the giant loop RNA has an unusual nucleotide composition, high in cytidine and low in guanine (25% A, 27% U, 39% C, and 9% G) (Hartley and Callan, 1978). An unusual nucleotide composition is also indicated by the fact that the DNA axis of the giant loops is not cut by the restriction endonuclease Hae III (Gould et al., 1976), whereas most loops are readily digested by this enzyme (a “four-cutter” that recognizes the sequence GGCC). The giant loops are cut by other restriction enzymes and by DNase I, indicating that their DNA axis is generally accessible to enzymes. One interpretation of the incorporation and restriction enzyme data is that the giant loops contain a simple, repeated sequence that happens to lack GGCC. The transcription of repeated sequences ("satellite DNA") on lampbrush chromosome loops is well documented by in situ hybridization (Varley et al., 1980; Diaz et al., 1981). It will be of great interest to identify the RNA sequences with which the L protein is associated in the giant loops.

Immunofluorescence microscopy on fixed somatic cells (Fig. 6) shows one to three (generally two) loci of high concentration of L in the nucleus in addition to a general localization between the (maximum) number of granules in somatic nuclei and the number of brightly staining lampbrush loci would suggest that the same loops are active in somatic and germinal nuclei.

L is a new type of hnRNP protein also in its amino acid sequence. One of the remarkable features of the sequence of L is that it contains two α amino acid domains only distantly related to the consensus sequence RNA binding domain (Dreyfuss et al., 1988; Bandzlijulis et al., 1989). We have been unable to identify any additional significant homologies between L and other RNA-binding proteins, including hnRNP proteins, except for some limited sequence similarity between the glycine-rich amino terminus of the L protein and the carboxy domain of the hnRNP A1. Further analysis of the relationship between protein sequence characteristics and the distribution of specific hnRNP proteins on hnRNA will probably advance from further studies on many other of the >20 proteins in immunopurified hnRNP complexes (Piñol-Roma et al., 1988) that must still be analyzed.

In summary, the distribution of the L protein in the cell, its association with the bulk of hnRNP complexes as well as with unique transcripts, and the primary structure of the L protein deduced from cDNA cloning, all indicate that L represents a new and unique type of hnRNP protein. The findings reported here and the availability of the antibodies and cDNA clones for L open the way for a number of exciting investigations. These include isolation and characterization of the transcripts of giant loops, and the general question of what are the signals that direct specific proteins, such as L, to specific loci on chromosomes.

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