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Original Article

Heterogeneous nuclear ribonucleoprotein complexes from *Xenopus laevis* oocytes and somatic cells

AUDREY MARCU, BHRAMDEO BASSIT, ROSALIE PEREZ and SERAFÍN PIÑOL-ROMA*

Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, USA

ABSTRACT HnRNP proteins have been implicated in most stages of cellular mRNA metabolism, including processing, nucleocytoplasmic transport, stability, and localization. Several hnRNP proteins are also known to participate in key early developmental decisions. In order to facilitate functional studies of these pre-mRNA- and mRNA-binding proteins in a vertebrate organism amenable to developmental studies and experimental manipulation, we identified and purified the major hnRNP proteins and isolated the hnRNP complex from *Xenopus laevis* oocytes and somatic cells. Using affinity chromatography and immunological methods, we isolated a family of >15 abundant single-stranded nucleic acid-binding proteins, which range in apparent molecular weight from ~20 kDa to >150 kDa, and with isoelectric points from <5 to >8. Monoclonal antibodies revealed that a subset of these proteins are major hnRNP proteins in both oocytes and somatic cells in culture, and include proteins related to human hnRNP A2/B1/B2 and hnRNP K. UV crosslinking in living cells demonstrated that these proteins bind poly(A)$^+$ RNA *in vivo*. Immunopurification using a monoclonal antibody to *X. laevis* hnRNP A2 resulted in the isolation of RNP complexes that contain a specific subset of single-stranded nucleic acid-binding proteins. The protein composition of complexes isolated from somatic cells and from oocyte germinal vesicles was similar, suggesting that the overall properties and functions of hnRNP proteins in these two cell types are comparable. These findings, together with the novel probes generated here, will also facilitate studies of the function of vertebrate RNA-binding proteins using the well characterized *X. laevis* oocyte and early embryo as experimental systems.

KEY WORDS: *hnRNP complex, hnRNP proteins,* Xenopus *oocytes, mRNA formation, RNA-binding proteins*

Introduction

Regulation of gene expression at the post-transcriptional level has been extensively documented for all stages of mRNA metabolism, ranging from pre-mRNA processing to nucleocytoplasmic transport, stability, localization, and translation of mRNA (Harford and Morris, 1997). It is widely accepted that the substrate for all of these processes is the RNA with its associated proteins, in the form of ribonucleoprotein (RNP) complexes, rather than naked RNA (Dreyfuss *et al.,* 1993). Therefore, a thorough understanding of RNA metabolism in the cell requires an understanding of the structure, composition, and dynamic changes of RNP complexes.

The association of RNA with proteins begins as soon as the RNA emerges from the transcription machinery (Dreyfuss *et al.,* 1993). In the case of RNAs transcribed by RNA polymerase II, the nuclear complexes of RNA with its specifically associated proteins are collectively known as heterogeneous nuclear RNP (hnRNP) complexes. Much of our knowledge of hnRNP complexes and their components has been provided by studies in human cells (e.g. Beyer *et al.,* 1977; Choi and Dreyfuss, 1984; Piñol-Roma *et al.,* 1988), in which hnRNP complexes comprise a set of 24 highly abundant RNA-binding proteins, termed hnRNP A1 (34kDa) through hnRNP U (120kDa; Piñol-Roma *et al.,* 1988). HnRNP proteins in actively growing cells are as abundant as histones, and as a family they have been implicated in all stages of mRNA formation (Dreyfuss *et al.,* 1993; Krecic and Swanson, 1999). An essential role for hnRNP proteins in early development has been shown in *Drosophila melanogaster*. For example, absence of the *Drosophila* hrp40 hnRNP protein from the germ line leads to the dorsoventral mutant *squid* (Kelley, 1993; Matunis *et al.,* 1994). Similarly, mutations in the essential *Drosophila*

Abbreviations used in this paper: NEPHGE, Non-Equilibrium pH Gradient Gel Electrophoresis; RNP, ribonucleoprotein; hnRNP, Heterogeneous Nuclear Ribonucleoprotein; RSB, Reticulocyte Standard Buffer; ssDNA, single-stranded DNA.

***Address correspondence to:** Dr. Serafín Piñol-Roma. Department of Biochemistry and Molecular Biology. Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1007, New York, NY 10029-6574, USA. Fax: +1-212-860-1174. e-mail: serafin.pinol-roma@mssm.edu

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Fig. 1. Affinity chromatography of *Xenopus laevis* proteins on ssDNA-cellulose. *Whole X. laevis A6 cell lysate proteins were fractionated on a ssDNA-cellulose column as described in the text. Proteins in the various fractions were resolved by SDS-PAGE and visualized by staining with silver. Lanes are as follows: Total, starting material; F.T., unbound (flow-through) fraction; Heparin, proteins in a wash with 1mg/ml heparin; 0.2 M and 2.0 M NaCl, proteins eluted with the indicated NaCl concentrations. Positions of molecular mass standards are shown on the left.*

hnRNP protein hrp48 cause developmental defects and affect specific splicing processes (Hammond *et al.,* 1997). While less is known about the role of hnRNP proteins in early vertebrate development, recent studies have shown that the hnRNP C proteins are required for postimplantation development in mice, whereas embryonic stem cells that do not express hnRNP C proteins are viable in culture (Williamson *et al.,* 2000). It is also apparent that many individual hnRNP proteins exert functions at more than one stage in mRNA formation (see Krecic and Swanson, 1999, and references therein). Dissection of specific functions of hnRNP proteins *in vivo* is therefore made all the more challenging because disruption of different functions may lead to similar phenotypes/defects in mRNA formation.

Xenopus laevis is often used as a model system for studies of the regulation of mRNA formation and metabolism. This organism provides a uniquely valuable experimental tool with which to address the function of RNA-binding proteins, because of the relative ease of manipulation of *Xenopus* oocytes at a scale amenable to biochemical analyses, the ease with which defined molecules and macromolecular complexes can be injected, and the thorough characterization of its development (e.g. Dawid and Sargent, 1988; Richter, 1999). In addition, *Xenopus laevis* provides a natural experiment to study the relationship between transcriptional activity (and thus formation of the substrate for RNA-binding proteins) and the function of hnRNP proteins, given the dramatic changes in overall transcription that occur during its early development (Newport and Kirschner, 1982). This is of particular interest given that the nucleocytoplasmic distribution of several hnRNP proteins is dependent on ongoing transcription by RNA polymerase II (Piñol-Roma and Dreyfuss, 1992).

HnRNP proteins exhibit an overall high affinity for single-stranded nucleic acids in general, including ssDNA (Pandolfo *et al.,* 1987; Piñol-Roma *et al.,* 1988; Matunis *et al.,* 1992). Moreover, hnRNP complexes can be isolated using a relatively simple and rapid immunopurification procedure with monoclonal antibodies to individual hnRNP proteins, under conditions that preserve protein-RNA as well as, presumably, protein-protein interactions (Choi and Dreyfuss, 1984; Piñol-Roma *et al.,* 1988; Matunis *et al.,* 1992). Here, we have taken advantage of these properties to identify, isolate, and produce monoclonal antibodies to *X. laevis* hnRNP proteins. More importantly, one of the monoclonal antibodies described here has enabled us to isolate the hnRNP complex from *X. laevis* somatic cells in culture and from oocyte nuclei, and to compare their protein composition. The isolation of the hnRNP complex from *X. laevis* (especially from oocytes), together with the availability of specific probes, opens the way for a number of experimental approaches to probe the function of hnRNP proteins in an experimentally tractable vertebrate organism.

Results

Isolation of single-stranded nucleic acid-binding proteins from Xenopus laevis

Previous studies have shown that, *in vitro*, most hnRNP proteins have a relatively high affinity for single-stranded nucleic acids in general, including ssDNA (Pandolfo *et al.,* 1987; Piñol-Roma *et al.,* 1988). Conversely, in a number of organisms it has been shown that the predominant proteins that bind ssDNA *in vitro* are, in fact, RNA-binding proteins *in vivo* (e.g. Piñol-Roma *et al.,* 1988; Matunis *et al.,* 1992). Thus, fractionation on ssDNA-cellulose provides a simple, fast, and reasonably specific method for large-scale isolation of RNA-binding proteins, including primarily hnRNP proteins. We took advantage of these general properties to identify and isolate hnRNP proteins from *Xenopus laevis*.

Fractionation on ssDNA-cellulose revealed a substantial number of proteins from *X. laevis* kidney epithelial cells (A6 line) that bound ssDNA with relatively high affinity and specificity (Fig. 1). Whole cell lysate proteins (Fig. 1, lane 'Total') were loaded onto ssDNA-cellulose at moderately low (100mM NaCl) ionic strength. We used whole cell lysates, rather than nuclear fraction proteins, for two main reasons: a) we found that a large number of RNA-binding proteins leak from the nucleus under various nucleocyto-

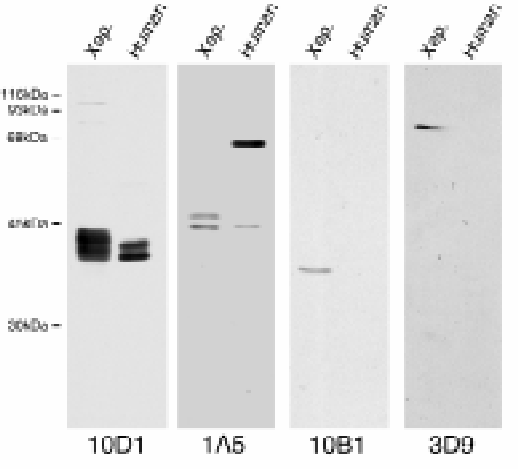

Fig. 2. Immunoblot analysis of *X. laevis* and human proteins with anti-*Xenopus* ssDNA-binding protein monoclonal antibodies. *Whole X. laevis lysate (lanes Xep.) and whole human HeLa cell lysate (lanes Human) proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated monoclonal antibodies. Positions of molecular mass standards are indicated on the left.*

plasmic fractionation procedures (data not shown), and b) we had an interest in isolating both mRNA- and hnRNA- binding proteins. The column was then washed with heparin as a non-specific competitor for proteins that may interact through non-specific electrostatic interactions with the sugar-phosphate backbone of ssDNA (Fig. 1, lane 'heparin'; Piñol-Roma *et al.,* 1988). Following a wash with 0.2M NaCl, bound proteins were eluted with 2.0M NaCl (Fig. 1, lane '2.0M NaCl'). Most proteins were in the unbound (flow-through) fraction, and a substantial number of proteins were also removed by heparin. Elution with 2.0M NaCl resulted in the release of a specific subset of proteins ranging in apparent molecular masses from ca. 20kDa and >150kDa. These include a prominent cluster between ca. 35kDa and 45kDa that is discussed further below. At this level of resolution and detection, >20 proteins are readily apparent in this fraction.

Monoclonal antibodies to X. laevis *single-stranded nucleic acid-binding proteins*

In order to facilitate the study of the candidate RNA-binding proteins described above, we used proteins eluted with 2.0M NaCl from ssDNA-cellulose as antigen for production of monoclonal antibodies. Several hybridomas were generated, which secreted antibodies specific for subsets of the proteins observed in Fig. 1. The specificity of these antibodies was determined by immunoblot analysis of whole *X. laevis* A6 cell lysates (Fig. 2). 10D1 binds a cluster of proteins of relative molecular masses between 35 and 45 kDa, which do not resolve well in total cell lysates (Fig. 2, '10D1, Xep.'). Upon long exposures of the blot, such as the one shown here, two additional bands can be seen at ca. 75kDa and 100kDa. 1A5 binds two different proteins around 45kDa, and a third protein with slightly faster mobility which is visible upon longer exposures of the film (e.g. see also Fig. 6). 10B1 and 3D9 appear to bind single proteins of ca. 35kDa and ca. 93kDa respectively. Two of these antibodies were also consistently found to bind specific proteins in whole human (HeLa) cell lysates: 10D1 bound two to three distinct polypeptides migrating at around 35 to 40kDa, and 1A5 bound a protein of ca. 68kDa (Fig. 2, lanes 'Human'). A minor 1A5-reactive human protein band observed at around 45kDa (Fig. 2, "1A5") is most likely a proteolytic product, since its presence and relative amounts vary greatly among samples (not shown). No significant reactivity of 3D9 or 10B1 towards HeLa proteins was observed by this analysis.

By immunofluorescence microscopy staining of A6 cells in culture, all the antibodies showed a predominantly nuclear location, which appeared to exclude nucleoli (Fig. 3). Overall, the signal was rather diffuse throughout the nucleoplasm, except for one of the antibodies, 3D9, which showed a more punctate nucleoplasmic staining (Fig. 3, panel '3D9, I.F.'). 1A5 showed occasional regions of higher apparent concentration of the antigen in some nuclei (see Fig. 3). 10B1 also showed weak diffuse cytoplasmic staining that was consistently slightly higher than background (Fig. 3, panel '10B1, I.F.'). Immunofluorescence staining of human (HeLa) cells with 10D1 and 1A5 also showed a predominantly nucleoplasmic staining (not shown).

Immunological relation of X. laevis *single-stranded nucleic acid-binding proteins with human hnRNP proteins*

When analyzed by two-dimensional gel electrophoresis, the proteins eluted from ssDNA-cellulose with 2M NaCl exhibit a wide range of isoelectric points, spanning virtually the entire range of separation in the first dimension, and a greater degree of complexity than revealed by SDS-PAGE alone (Fig. 4, panel 'Total protein'). In particular, the most prominent proteins migrate as a cluster in the region between ~35 to ~45 kDa, and resolve into >14 different polypeptides. This complexity may result both from the presence of distinct polypeptides, including possibly variations due to alternative splicing, and from post-translational modifications resulting in isoelectric variants of individual proteins (as has been shown for several hnRNP proteins; see Dreyfuss *et al.,* 1993).

Because of their stronger reactivity towards *X. laevis* proteins, and their reactivity with human proteins, we focused our subsequent studies on the 10D1 and 1A5 monoclonal antibodies. Immunoblot analysis of two-dimensional gels revealed that 10D1 binds a subset of the ssDNA-binding proteins that migrate between

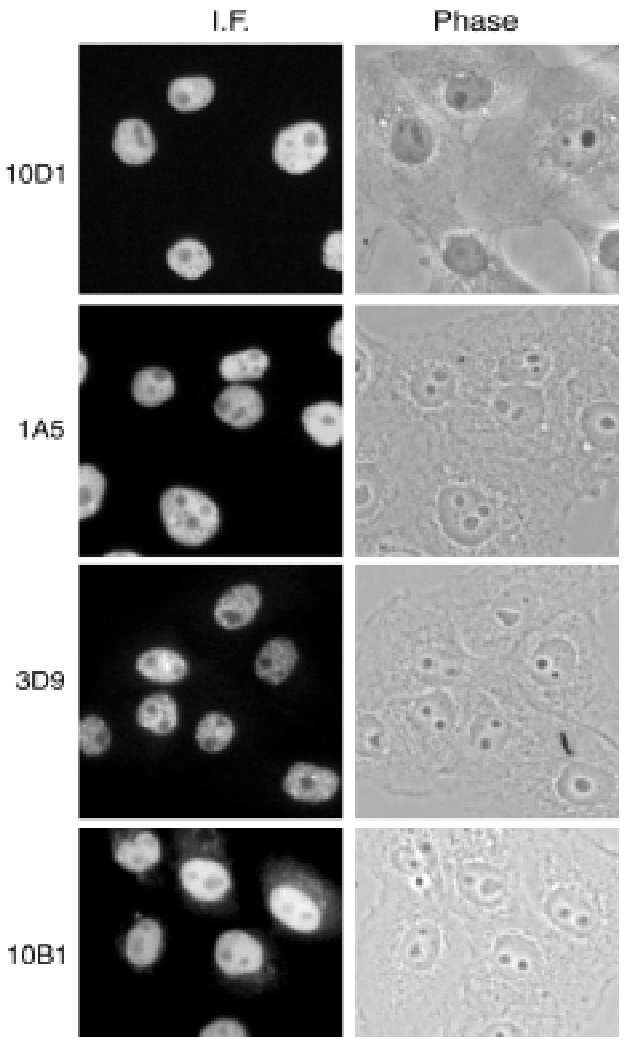

Fig. 3. Immunofluorescence microscopy staining of *X. laevis* cells with monoclonal antibodies to ssDNA-binding proteins. *X. laevis kidney epithelial (A6) cells grown on glass slides were fixed, permeabilized, and immunostained with the indicated monoclonal antibodies. Representative fields of cells are shown in each case, together with the corresponding phase microscopy image. Bar, 10 μm.*

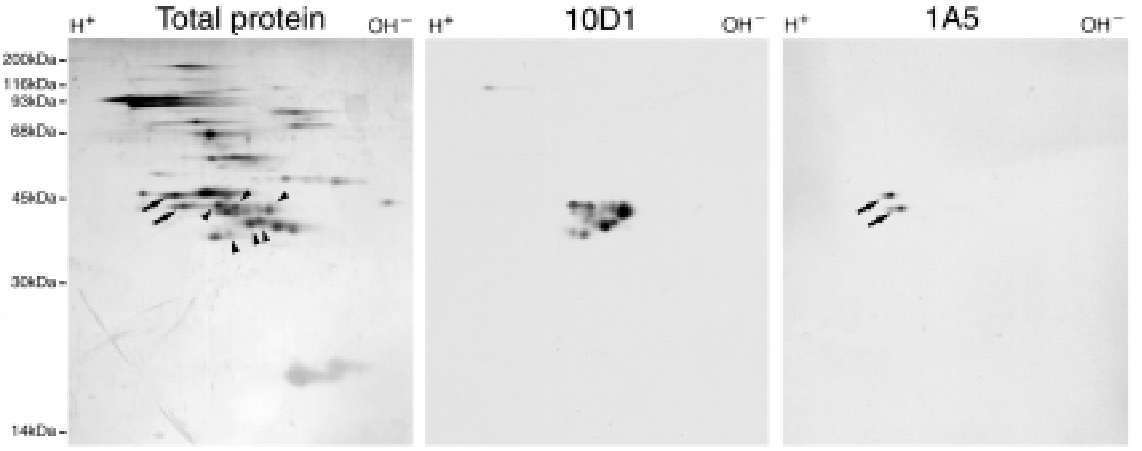

Fig. 4. Two-dimensional gel electrophoresis and immunoblot analysis of *X. laevis* ssDNA-binding proteins. *Proteins from the 2.0 M NaCl eluate shown in Fig. 1 were resolved by two-dimensional gel electrophoresis, using NEPHGE in the first dimension and SDS-PAGE in the second dimension. The separated proteins were visualized by silver staining (left panel), by immunoblotting with 10D1 (middle panel), or by immunoblotting with 1A5 (right panel). Arrowheads in the left panel point to proteins reactive with the 10D1 monoclonal antibody. Arrows in the left and right panels point to the position of the 1A5-reactive proteins. Positions of molecular mass standards are indicated on the left.*

35 and 40kDa in the gel, and with isoelectric points near neutral values (Fig. 4, panel '10D1'). The specific proteins bound by the antibody are identified in the corresponding silver-stained gel with arrowheads. While 10D1 binds most of the proteins in this region, there are a number of prominent proteins in this cluster that are not bound by this antibody. Identical results were obtained in immunoblots using whole cell lysates (data not shown). Thus, all the proteins bound by 10D1 are among those that bind to the ssDNA-cellulose column. Similarly, 1A5 reacts with a doublet of more acidic proteins migrating at ca. 45kDa, which are also readily identifiable in the silver-stained gel (Fig. 4, arrows). Again, identical results were observed in immunoblot analyses of whole cell proteins (data not shown). Since the proteins bound by 10D1 and 1A5 are among those that remain bound to the ssDNA column even after extensive washes with heparin, the interaction of these proteins with single-stranded nucleic acids appears to be specific.

The properties described above are characteristic of many known hnRNP proteins. Therefore, we asked whether the proteins bound by 10D1 and 1A5 in human cells correspond to any previously identified hnRNP proteins. The hnRNP proteins in HeLa cells have been characterized in detail by two-dimensional gel electrophoresis, and most of them bind strongly to ssDNA-cellulose (Piñol-Roma *et al.,* 1988). HeLa cell ssDNA-binding proteins were purified from nuclear extracts as done above for *X. laevis* proteins. Proteins eluted with 2.0M NaCl were resolved by two-dimensional gel electrophoresis and analyzed by immunoblotting with 10D1 and 1A5 (Fig. 5). The total protein composition of this fraction, in which individual hnRNP proteins are readily identifiable, was also visualized by silver staining (for reference purposes, the positions of hnRNP A1, A2, B1, B2, and K are indicated in Fig. 5). Immunoblotting with 10D1 shows that this antibody reacts strongly with hnRNP A2, B1, and B2 (Fig. 5, panel '10D1'). No reactivity with the highly related hnRNP A1 is observed at this level of detection. It is interesting to note that hnRNP B1 is identical to hnRNP A2 except from an insertion of 12 amino acids near the amino terminus of the protein, resulting most likely from inclusion of a small exon through alternatively splicing (Burd *et al.,* 1989). A similar analysis shows that 1A5 binds human hnRNP K (Fig. 5, panel '1A5').

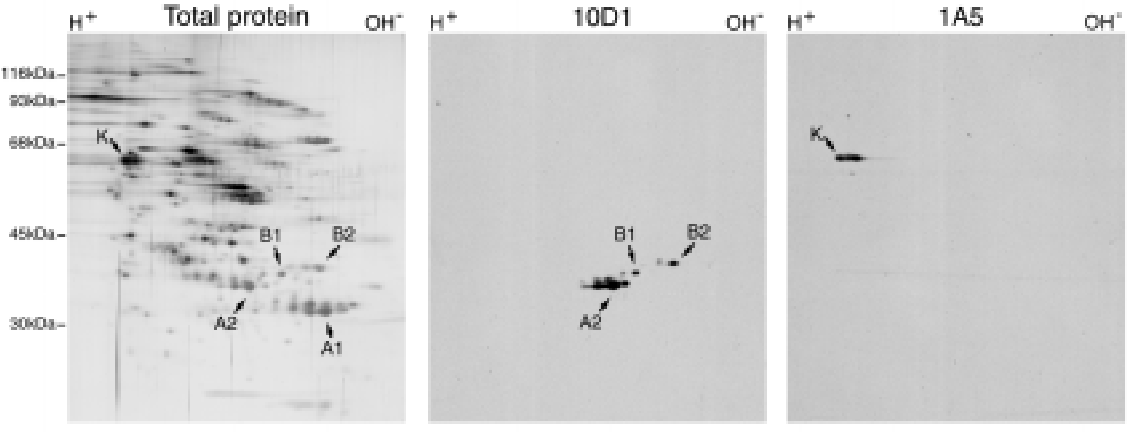

Fig. 5. Identification of human hnRNP proteins as antigens for 10D1 and 1A5. *HeLa cell nuclear extract was fractionated by ssDNA-cellulose chromatography, and proteins eluted with 2.0 M NaCl were resolved by two-dimensional gel electrophoresis. Proteins were visualized by silver staining (left panel), or by immunoblotting with 10D1 (middle panel) or 1A5 (right panel). The positions of hnRNP A1, A2, B1, B2, and K, are indicated on the silver-stained gel.*

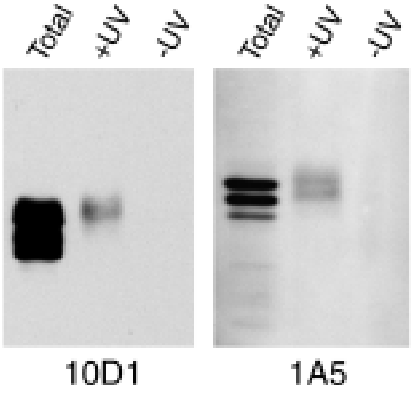

Fig. 6. Immunoblot analysis of proteins crosslinked to poly(A)⁺ RNA *in vivo*. *Living X. laevis A6 cells were exposed to ultraviolet light, and poly(A)⁺ RNA was then isolated with its covalently bound proteins. Proteins were released from the crosslinked complexes with RNase digestion, resolved by SDS-PAGE, and probed with the indicated antibodies. Lanes are labeled as follows: Total: whole A6 cell proteins without crosslinking and oligo(dT) selection; +UV: oligo(dT)-selected complexes from cells exposed to UV light; -UV: control experiment carried out as shown in the +UV lanes except that exposure of the cells to UV light was omitted.*

Association of proteins and poly(A)⁺ RNA in vivo

The characteristics of the proteins identified above, namely binding to ssDNA, nuclear location, and immunological relatedness to known human hnRNP proteins, indicate strongly that they are *bona fide X. laevis* hnRNP proteins. To test this directly, we induced covalent crosslinks between RNA and its associated proteins *in vivo* by exposing living *X. laevis* A6 cells to ultraviolet light. Under these conditions, only proteins that are in direct contact with RNA *in vivo* can become covalently crosslinked to it. This allows selection of RNA with covalently bound proteins under protein-denaturing conditions, in order to eliminate adventitious association of proteins with the RNA during fractionation (Dreyfuss *et al.,* 1984; van Eekelen *et al.,* 1981; Mayrand *et al.,* 1981). *X. laevis* A6 cells were irradiated with UV light, and poly(A)-containing RNA was isolated by affinity chromatography on oligo(dT) cellulose after heating in the presence of SDS and ß-mercaptoethanol. The crosslinked RNPs were then digested with RNase in order to release covalently bound proteins, which were subsequently resolved by SDS-PAGE. Figure 6 shows immunoblot analysis of crosslinked proteins using the 10D1 and 1A5 antibodies. Both antibodies react with proteins that are among those crosslinked to RNA *in vivo* (lanes '+UV'). This is further confirmed by the characteristic slight mobility shift of the corresponding bands as well as their more diffuse appearance, due to residual crosslinked nucleotides that resist RNase digestion. The specificity of crosslinking is underscored by the absence of signal in cells that had not been exposed to ultraviolet light (lanes '-UV'). At this level of detection, and because of alterations in mobility of the proteins due to crosslinking, we cannot determine with certainty how many and specifically which of the 10D1-reactive proteins are crosslinked to the RNA.

Protein composition of hnRNP complexes isolated from X. laevis *somatic cells in culture and from oocytes*

The analyses described above indicate strongly that 10D1 binds *X. laevis* hnRNP A2. To confirm this, we transcribed and translated *X. laevis* hnRNP A2 *in vitro* from the corresponding cDNA (Good *et al.,* 1993), and tested its binding to 10D1 by immunoprecipitation. The results, shown in Fig. 7, confirm that 10D1 binds the *X. laevis* hnRNP A2 (lane '10D1'). By contrast, no labeled protein was immunoprecipitated with a monoclonal antibody to human hnRNP A1 (Fig. 7, lane '4B10'), thus confirming the specificity of the 10D1 antibody towards hnRNP A2.

Given the reactivity of 10D1 with *X. laevis* proteins and its specificity towards *bona fide* hnRNP proteins, we asked whether it could be used to isolate hnRNP complexes from *X. laevis*, as has been done in the past with other antibodies to isolate human (Choi and Dreyfuss, 1984; Piñol-Roma *et al.,* 1988) and *D. melanogaster* (Matunis *et al.,* 1992) hnRNP complexes. To do so, A6 cell lysate was incubated briefly with 10D1 immobilized on protein A-Sepharose beads, in isotonic buffer conditions that preserve most protein-RNA interactions. After extensive washing, immunoadsorbed complexes were eluted and the proteins were resolved by two-dimensional gel electrophoresis. Figure 8 shows that, in addition to the proteins bound directly by 10D1, a number of additional polypeptides co-purify with the 10D1 antigens. Many (but not all) of these co-purified polypeptides (indicated with small arrows) co-migrate with proteins that bound to ssDNA (compare with Fig. 4). Among the 15 to 20 proteins that co-purify in the complexes, the 1A5 antigens were readily identifiable by virtue of their electrophoretic mobility (compare with Fig. 4), and their identity was confirmed by immunoblot analysis (not shown). Similarly, another hnRNP protein, hnRNP L, was identified by immunoblotting with the 4D11 monoclonal antibody (Piñol-Roma *et al.,* 1989) and is indicated here (Fig. 8, panel '10D1-Triton'). Several relatively small (<30kDa), primarily basic polypeptides are apparent. Although the precise identity of these proteins is not known, their mobility is consistent with that of snRNP proteins, which have also been observed by others in association with hnRNP A2/B1 in mammals (Kamma *et*

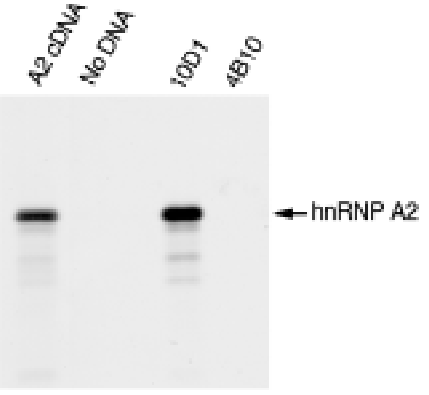

Fig. 7. Binding of 10D1 to *X. laevis* hnRNP A2 transcribed and translated *in vitro*. *X. laevis hnRNP A2 was transcribed from the corresponding cDNA and translated in vitro in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. The translated protein was subsequently immunoprecipitated using either 10D1 or the anti-hnRNP A1 4B10 monoclonal antibody, as indicated. Total translation reaction equivalent to one-fifth of the material used for immunoprecipitation was resolved on the same gel for comparison (lane 'A2 cDNA'). The translation products were visualized by autoradiography. Lane 'No DNA': total translation product from an identical reaction performed in parallel in which no exogenous DNA was added.*

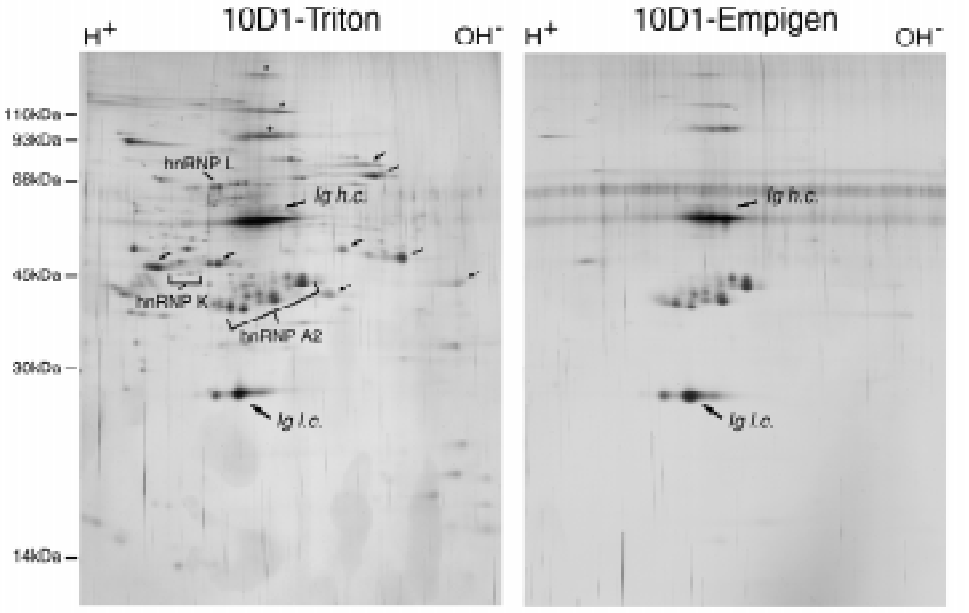

Fig. 8. Immunopurification of *X. laevis* hnRNP complexes from somatic cells. *Immunopurifications with the 10D1 antibody were carried out from X. laevis cell lysate prepared in isotonic buffer containing 0.5% Triton X-100 (panel TNX-100) or in the presence of the ionic detergent Empigen BB (panel Empigen). Immunopurified complexes were resolved by two-dimensional gel electrophoresis, and proteins were visualized by silver staining. The positions of known hnRNP proteins (determined by immunoblot analyses not shown) are indicated. Proteins that also bind ssDNA are indicated with arrows. Ig h.c. and Ig l.c. refer to heavy and light chains, respectively, of the 10D1 antibody. Asterisks indicate proteins originating also from the antibody preparation. Positions of molecular mass standards are indicated on the left.*

al., 1999). The specificity of the association of the observed proteins with hnRNP A2 is underscored by the fact that several of the predominant proteins that were found to bind to ssDNA-cellulose are absent from the immunopurified complexes (compare Fig. 8 with Fig. 4). Furthermore, when the immunopurification was carried out in the presence of the ionic detergent Empigen BB, which disrupts most protein-protein as well as protein-RNA interactions (Choi and Dreyfuss, 1984), only the 10D1 antigens were immunopurified (Fig. 8, panel "Empigen"), thus confirming the specificity of the antibody. The proteins not directly bound by 10D1 dissociate from the immunopurified complexes upon digestion with RNase (not shown), indicating that RNA is indeed a component of these complexes and that it is required for the stable association and co-isolation of the observed proteins.

Because of the unique experimental system afforded by *Xenopus laevis* oocytes for studying RNA-binding proteins and RNA metabolism in general, we asked whether 10D1 could also be used for the specific isolation of hnRNP complexes from oocytes. Furthermore, the ability to specifically isolate these complexes would enable us to determine the extent to which oocyte hnRNP complexes resemble those in somatic cells. A similar experiment to that shown in Fig. 8 was carried out in order to isolate hnRNP complexes from manually dissected stage VI oocytes. As shown in Fig. 9, immunopurification with 10D1 from oocyte germinal vesicles isolates the corresponding antigens, together with additional proteins which exhibit similar electrophoretic mobility characteristics to those of hnRNP complexes from A6 cells (Fig. 9, 'Nucleus'). With few exceptions, all of the proteins observed in the oocyte com-

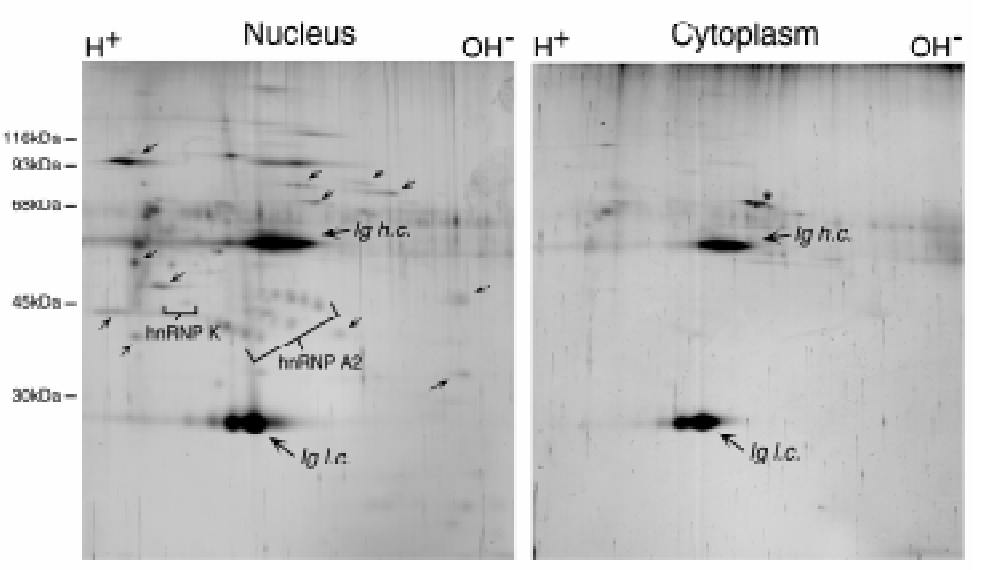

Fig. 9. Immunopurification with 10D1 from *Xenopus* oocyte nuclear and cytoplasmic fractions. *X. laevis oocytes (ca. 20 oocytes) were manually dissected into nuclear and cytoplasmic fractions, and immunopurifications were carried out from each fraction with the 10D1 antibody as described in Fig. 8. Proteins in the immunopurified complexes were resolved by two-dimensional gel electrophoresis and visualized by silver staining also as in Fig. 8. Left panel: complexes immunopurified from the nuclear fraction. The positions of hnRNP A2/B1/B2 and hnRNP K are indicated, as are the positions of the heavy and light chains of the antibody used for immunopurification. Arrows point to additional proteins in the complex that co-migrate with those observed in complexes immunopurified from A6 cells (compare with Fig. 8). Right panel: immunopurification from the cytoplasmic fraction of an identical number of oocytes as in the left panel.*

plexes co-migrate with those observed in somatic complexes (see arrows in Fig. 9) and vice versa: most of the proteins observed in the somatic complexes are present in the oocyte hnRNP complexes. Few if any proteins are immunopurified with 10D1 from the cytoplasmic fraction of an identical number of oocytes (Fig. 9, 'Cytoplasm'), indicating that at steady-state levels these proteins are primarily nuclear. Only upon overloading of the gels are any of the 10D1 antigens detectable in the cytoplasmic fraction (not shown). We note that a small amount of an unidentified oocyte protein (marked with an asterisk) is immunopurified from the cytoplasmic fraction. Similar complexes to those shown here from the nuclear fraction are also immunopurified with 10D1 from whole oocytes (not shown), thus underscoring the specificity of the isolation.

Discussion

The work described here aimed at isolating the hnRNP complex from *Xenopus laevis*, identifying the major pre-mRNA- and mRNA-binding proteins in this organism, and comparing the composition of hnRNP complexes from somatic cells and oocytes. Our motivation behind studying hnRNP complexes in *X. laevis* lies in the unique properties of this organism as an experimental model system for studies of RNA metabolism and of the function of RNA-binding proteins *in vivo* in vertebrates. Among the advantages offered by *Xenopus laevis* are the size and relative ease of manipulation of its oocytes, and the rather extensive characterization of its early developmental events (e.g. Dawid and Sargent, 1988). Using affinity chromatography on ssDNA and production of monoclonal antibodies, we have identified several of the hnRNP proteins in *X. laevis* and, more importantly, we have immunopurified the hnRNP complex from this organism. Similar approaches have been used successfully in the past for purifying hnRNP proteins and isolating hnRNP complexes from human (Choi and Dreyfuss, 1984; Pandolfo *et al.,* 1987; Piñol-Roma *et al.,* 1988) and *Drosophila melanogaster* (Matunis *et al.,* 1992) cells in culture. Few *X. laevis* proteins with significant sequence similarities to human hnRNP proteins have been identified to date (e.g. Kay *et al.,* 1990; Good *et al.,* 1993; Siomi *et al.,* 1993; Preugschat and Wold, 1988; Eckmann and Jantsch, 1997) and, to our knowledge, this is the first report of the specific isolation and comparison of hnRNP complexes from *X. laevis* somatic cells and oocytes.

The proteins bound by the 10D1 and 1A5 antibodies are *bona fide* hnRNP proteins as defined by the nuclear location of their corresponding antigens (Fig. 3) and by the fact that these proteins are bound to poly(A)$^+$ RNA *in vivo* (Fig. 6). Furthermore, both of these antibodies bind known hnRNP proteins in human cells (Fig. 5). We have shown that 10D1 binds the *X. laevis* hnRNP A2 protein (Fig. 7). The presence of multiple 10D1-reactive polypeptides both in somatic cells in culture and in oocytes is consistent with the existence of several isoforms of the *X. laevis* hnRNP A2 generated by alternative splicing (Good *et al.,* 1993), as is the case with hnRNP A2 and B1 in human cells (Burd *et al.,* 1989). The antigens for 1A5 in *X. laevis* are likely to correspond to human hnRNP K/J, which would be consistent with the differences in amino acid sequence of hnRNP K in these two organisms, which in *X. laevis* contains two large deletions (removing ca. 66 amino acids) as compared to its human counterpart (Siomi *et al.,* 1993).

More importantly, the 10D1 monoclonal antibody has allowed us to isolate and compare hnRNP complexes from *X. laevis* somatic cells in culture and oocytes (Figs. 8 and 9). The conclusion that the isolated complexes are indeed hnRNP complexes is based on the nuclear location and demonstrated RNA-binding activity of several of their constituents, their association with at least two other hnRNP proteins (the 1A5 antigen and hnRNP L), and their sensitivity to RNase digestion (A.I.M. and S.P.R., unpublished observations). Furthermore, the specificity of the additional associated proteins is underscored by the absence in these complexes of several major single-stranded nucleic acid-binding proteins observed by affinity chromatography on ssDNA (compare Figs. 4 and 8). Overall, the characteristics of the *X. laevis* hnRNP complexes are similar to those of their human and *D. melanogaster* counterparts with regards to their protein composition, in that: a) they comprise a group of between 15 and 25 polypeptides that exhibit a wide range of apparent molecular masses and isoelectric points; b) as a group, they show rather strong binding activity towards ssDNA; c) their association with one another in the complex requires the presence of RNA; d) their association with the hnRNP complex is specific in that other proteins that also bind strongly to ssDNA are not found in the complex. 10D1 can also immunopurify the hnRNP complex from human cells (S. Mili and S.P.R., unpublished observations). We have not been able to carry out similar immunopurifications with the 1A5 antibody (data not shown), which could be due to inaccessibility of the epitope to the antibody, or to disruption of the hnRNP complexes upon binding of 1A5 to its antigen.

An important finding from this work is that hnRNP complexes immunopurified from somatic (kidney epithelial) cells in culture and from oocytes are very similar, but not identical, in their protein composition. This indicates that the overall characteristics of complexes of pre-mRNA and mRNA with their bound proteins are maintained among these different cell types. From an experimental point of view, this also suggests that somatic cells in culture and oocytes likely provide comparable systems in which to study general RNA metabolic events, at least with regards to the participation of hnRNP proteins in these processes. A small subset of the proteins apparent in the complexes immunopurified from somatic cells appear to be absent from complexes immunopurified from oocytes. At this stage, we do not know whether this is due simply to limitations in sensitivity of the detection, to partial disruption of the complexes during their isolation from oocytes, or to *bona fide* cell type-specific differences between the two complexes. We note that the primarily nuclear location of the hnRNP proteins observed in the oocyte does not imply nuclear restriction of these proteins. It has been shown that some hnRNP proteins shuttle rapidly between the nucleus and the cytoplasm, but the steady-state levels of these proteins in the cytoplasm are very low probably due to their rapid re-import into the nucleus (Piñol-Roma and Dreyfuss, 1992).

The identification of *X. laevis* hnRNP proteins, the availability of specific probes to them, and the ability to immunopurify the *X. laevis* hnRNP complex, will facilitate the study of endogenous hnRNP complexes in *X. laevis* oocytes and embryos, as well as those RNP complexes assembled on RNA molecules introduced artificially into *X. laevis* oocytes for studies of mRNA formation (Dawid and Sargent, 1988; Richter, 1999). This will also facilitate the use *X. laevis* oocytes to probe the specific function(s) of individual RNA-binding proteins in mRNA formation and in early

development. It is noteworthy that, as a group, many of the *D. melanogaster* hnRNP proteins exhibit hallmark sequence features of human hnRNP proteins. However, the identification of specific *Drosophila* counterparts to individual human hnRNP proteins has been difficult. In this regard, it is likely that *X. laevis* will provide a more useful model system for studying the function of individual vertebrate hnRNP proteins with clearer homology to specific human hnRNP proteins (Preugschat and Wold, 1988; Kay *et al.,* 1990; Good *et al.,* 1993; Siomi *et al.,* 1993). Furthermore, these studies set the stage for following the distribution and intracellular location of specific hnRNP proteins in early developmental stages. This is of particular interest given that oocyte nuclear proteins follow specific temporal patterns of re-import into embryonic nuclei after fertilization (Dreyer, 1987). In the case of hnRNP proteins, this could be of importance in regulating their access to nuclear components and/or modulating their likely cytoplasmic functions. In this regard, it will be of interest to determine the extent to which nuclear location of hnRNP proteins in early *X. laevis* embryos is coupled to the onset of embryonic transcription at the midblastula transition (Newport and Kirschner, 1982), since in somatic cells it has been shown that RNA polymerase II-dependent transcription is required for efficient nuclear accumulation of a subset of hnRNP proteins (Piñol-Roma and Dreyfuss, 1992).

Finally, hnRNP proteins are known to function at multiple stages in mRNA formation (see Krecic and Swanson, 1999), but little is known as to the extent to which a specific temporal order of assembly of hnRNP proteins onto pre-mRNA and mRNA is required for these various functions. There is accumulating evidence, nonetheless, that association of hnRNP proteins with pre-mRNA and/or mRNA needs to occur in the nucleus for the subsequent function of hnRNP proteins in the cytoplasm. For example, binding of hnRNP proteins to RNA in the nucleus has been proposed to play a central role in masking of mRNAs in the cytoplasm of oocytes (Bouvet and Wolffe, 1994). More recently, studies have provided evidence that association of mRNA with hnRNP proteins in the nucleus plays a determining role in subsequent asymmetric localization of mRNAs in the cytoplasm, with its corresponding developmental consequences (Hoek *et al.,* 1998; Cote *et al.,* 1999; Lall *et al.,* 1999). By probing the function of specific hnRNP proteins *in vivo*, in a system in which different stages in mRNA formation can be segregated experimentally, it will be possible to define in detail the specific role of hnRNP proteins at each of these stages, as well as the extent to which hnRNP proteins may participate in coordinating different steps in the formation of mRNA.

Materials and Methods

Cell culture

Xenopus laevis A6 cells were grown in monolayer culture at 25°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (P.-S.), and 20% H_2O. Human (HeLa) cells grown in monolayer culture were maintained in DMEM, supplemented with 10% FBS and 1% P.-S. HeLa cells grown in suspension culture were purchased from the Cell Culture Center (Minneapolis, MN).

Cell fractionation

Fractionation of A6 cells was performed by a modification of previously described procedures (Piñol-Roma *et al.,* 1988). All steps were carried out at 0°-4°C. Cells grown in monolayer culture to subconfluent density were washed with PBS and collected with a cell scraper in RSB-100 (10 mM Tris, pH 7.4; 100 mM NaCl; 2.5 mM $MgCl_2$) containing 0.5% Triton X-100, 0.5% aprotinin, and 1 μg/ml each of leupeptin and pepstatin A. The cells were lysed by sonication, three times for 5 sec. each, using a microtip sonicator (model XL2015; Heat Systems, Farmingdale, NY) set at scale 2.5. The sonicate was layered over a 30% (w/v) sucrose cushion in RSB-100, and centrifuged at 4,000 x g for 15 min. The supernatant fraction was used for all experiments unless otherwise indicated. HeLa cell nuclear extract was prepared from cells grown in suspension culture as described previously (Dignam *et al.,* 1983). Stage VI *Xenopus laevis* oocytes (Dumont, 1972) were obtained from the *Xenopus* oocyte core facility at the Mount Sinai School of Medicine, and manually dissected in 5:1 buffer (Gall *et al.,* 1991). Germinal vesicles and cytoplasmic fractions were collected separately, and Triton X-100 was added to a final concentration of 0.5% (v/v). The samples were sonicated, clarified by centrifugation, and used for immunopurification.

Affinity chromatography on single-stranded DNA

ssDNA cellulose (US Biochemicals, Cleveland, OH) was equilibrated in 50 mM sodium phosphate, pH 7.4, containing 100 mM NaCl. Soluble cellular proteins were loaded on the column in the same buffer, and bound proteins were eluted with the indicated NaCl concentrations in 50 mM NaPhosphate buffer, pH 7.4. Where noted, the column was washed with 100 mM NaCl containing 1mg/ml heparin (Sigma) as a nonspecific competitor of the ssDNA (Piñol-Roma *et al.,* 1988).

Production of monoclonal antibodies

Monoclonal antibodies were obtained by immunization of a Balb/c mouse with *X. laevis* proteins purified by affinity chromatography on ssDNA-cellulose. Hybridoma production and screening were performed as previously described (Piñol-Roma *et al.,* 1988). All hybridoma cell lines were cloned at least three times by limiting dilution. Hybridoma-conditioned serum-free medium (HyQ-CCM1; Hyclone) was used as the source of antibody for immunofluorescence microscopy, immunopurification, and immunoblot analyses.

Gel electrophoresis and immunoblot analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analyses were carried out as previously described (Piñol-Roma *et al.,* 1988). For SDS-PAGE, the separating gel had a final acrylamide concentration of 12.5%. Two-dimensional gel electrophoresis was performed as described by O'Farrell *et al.* (1977). Separation in the first dimension was performed by non-equilibrium pH gradient gel electrophoresis (NEPHGE) using a pH 3-10 ampholite gradient for 4 h at 400 volts, and the second dimension was resolved by SDS-PAGE. Proteins were visualized by silver staining (Morrissey, 1981). Immunoblot analyses were performed with undiluted hybridoma-conditioned culture medium. Total cell lysate from approximately 10^5 cells was loaded in each lane for determining antibody specificity. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Organon-Teknika), followed by enhanced chemiluminescence (ECL; Amersham).

Immunopurification of hnRNP complexes

For immunopurification of hnRNP complexes (Choi and Dreyfuss, 1984; Piñol-Roma *et al.,* 1988), antibodies were pre-bound to protein A-Sepharose (Pharmacia) for 1 hour at 4°C, with constant rocking. Antibody-coated resin was then washed three times with RSB-100 containing 0.5% Triton X-100, and incubated with cell lysate derived from ca. 5 x 10^6 A6 cells, or material derived from ~20 oocytes, at 4°C for 15 minutes. The resin was washed 5 times with RSB-100 containing 0.5% Triton X-100, and bound complexes were eluted with NEPHGE sample buffer. Antibody specificity was confirmed by performing immunopurifications in the presence of the ionic detergent Empigen BB (Calbiochem) at 1%, 1 mM EDTA, and 0.1 mM DTT (Choi and Dreyfuss, 1984).

Ultraviolet light-induced crosslinking of RNA to proteins in vivo and analysis of crosslinked complexes

Crosslinking of proteins to RNA *in vivo* by UV light irradiation of cells on culture dishes, followed by selection of crosslinked complexes by oligo-dT chromatography, was carried out essentially as previously described (Dreyfuss *et al.,* 1984). Proteins were released from crosslinked complexes by digestion with RNase A at 50 μg/ml for 1 h at 30°C, resolved by SDS-PAGE, and analyzed by immunoblotting with the indicated monoclonal antibodies.

In vitro translation and immunopurification of Xenopus laevis hnRNP A2

X. laevis hnRNP A2 (kind gift from Dr. I. Dawid; Good *et al.,* 1993) was transcribed from the corresponding cDNA with SP6 RNA polymerase and translated *in vitro* using a coupled transcription-translation system (Promega Biotech), in the presence of ^{35}S-methionine. The translated protein was immunopurified using the 10D1 monoclonal antibody or, as a control, the 4B10 (anti-human hnRNP A1) monoclonal antibody. As a control, identical reactions were performed in parallel without added DNA.

Immunofluorescence microscopy

Cells grown on glass slides were fixed with 2% formaldehyde in PBS for 20 min. at room temperature, and permeabilized with acetone at -20°C for 3 min. Fixed and permeabilized cells were incubated with hybridoma-conditioned culture medium, diluted 1:5 with PBS containing 3% BSA, for 1 h. at room temperature in a humidified chamber. After a wash in PBS, the cells were incubated with FITC-labeled goat anti-mouse IgG (1:100 dilution; Organon-Teknika-Cappel) for 30 min., washed in PBS, and mounted with glass coverslips. Staining was observed with a Zeiss Axiophot microscope (Carl Zeiss, Thornwood, NY) using a 40X Plan-Neofluar objective.

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