Heterogeneous nuclear ribonucleoprotein particles and the pathway of mRNA formation
Gideon Dreyfuss, Maurice S. Swanson and Serafin Piñol-Roma

Heterogeneous nuclear ribonucleoprotein (hnRNP) particles, the structures that package hnRNA, are one of the major constituents of the nucleus. Recent work has led to the immunopurification of hnRNP particles and the identification of their proteins, and demonstrated a role for hnRNP proteins in mRNA splicing. The molecular cloning and sequencing of cDNAs for RNP proteins made possible the discovery of a conserved RNA-binding domain and a RNP consensus sequence.

In eukaryotic cells, messenger RNAs (mRNAs), the functional translatable intermediates of gene expression, are generally formed by extensive processing of primary gene transcripts. Historically, the primary gene transcripts were discovered in higher eukaryotes and termed heterogeneous nuclear RNAs (hnRNAs). hnRNAs can be distinguished from other RNAs on the basis of their size, their subcellular compartmentation, and the characteristics (e.g. antibiotic sensitivity) of the RNA polymerase that transcribes them. The terms hnRNA and pre-mRNA are often used interchangeably, although it is possible that only a subpopulation of hnRNAs are actually mRNA precursors. Typically, pre-mRNAs contain 5' cap structures (m7Gppp), polyadenylated tails, and the majority of them contain intervening sequences that are later spliced out. Little is known about the ensuing events except that the RNA is translocated through nuclear pores and that spliced mRNAs accumulate in the cytoplasm. One of the ultimate goals of molecular and cell biology is to understand all of these processes in terms of both molecular detail and cellular topology.

Work from numerous laboratories over the past 20 years has revealed that hnRNAs in cells do not normally occur as naked polynucleotides but are found in complexes with specific proteins (reviewed in Refs 1 and 2), which are termed hnRNP complexes or hnRNP particles. Interest in hnRNP complexes stems from the fact that the processing of pre-mRNA probably takes place on these ribonucleoprotein complexes. Therefore, to understand how the post-transcriptional portion of the pathway of expression of genetic information operates in the cell, we need to learn more about the ribonucleoprotein complexes.

The proteins of hnRNP particles
hnRNP particles are one of the major components of the nucleus. The proteins of the hnRNP particles are as abundant as histones in the nucleus of growing cells and comprise ~80% of the mass of hnRNP particles. The association of hnRNPs with hnRNP proteins begins as the hnRNA is still a nascent transcript and persists throughout its nuclear residency. Biochemical analyses of nuclear fractions containing hnRNA or hnRNA fragments obtained by velocity sedimentation revealed a group of proteins (of about 30-43 kDa) as consistent components of hnRNP complexes3-7. These proteins are detected by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as six bands and classified into three groups of proteins: A, B and C (Ref. 3). Thus it was thought that hnRNPs were composed predominantly of these six proteins. Although numerous additional proteins of higher molecular mass have been frequently observed in hnRNP fractions6-7 there has been no consensus about their authenticity. This is due to the intrinsic limitations of the velocity sedimentation method; it does not afford complete separation of hnRNP particles from other nuclear components, and non-specific association of proteins with the hnRNA after cell fractionation can not be ruled out.

A more stringent definition of genuine hnRNP proteins has been provided by photochemical (UV light-induced) covalent cross-linking of proteins to RNA in vivo, which allows identification of proteins that are bound to the RNA in the cell10. This method relies on the fact that UV light photo-activates RNA and converts it to an extremely reactive, short-lived molecule which reacts virtually indiscriminately with other molecules (including proteins) that are in direct contact with it. In effect this is photo-affinity labeling of the RNA-binding proteins in vivo. The cross-linked hnRNA–protein and mRNA–protein complexes can then be isolated from the nuclear and cytoplasmic fractions, respectively, after boiling in SDS and mercaptoethanol, by affinity chromatography on the oligo(dT)-cellulose, which selects them through binding to the poly(A) tail. These protein-denaturing

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Fig. 1. The experimental strategy used for the identification and production of monoclonal antibodies to RNA-binding proteins in vivo10. Step (1) protein–RNA cross-linking; step (2) RNA selection; and step (3) immunization. C, cytoplasmic fraction; N, nuclear fraction.
conditions ensure that only proteins which are covalently linked to the RNA are purified with it, thus eliminating non-specific associations of proteins with the RNA. The cross-linked proteins can be released from the complexes by digestion with RNases and analysed by SDS-PAGE. These studies showed numerous proteins, including the proteins that correspond in SDS-PAGE mobility to A, B and C proteins. This provided further evidence for the authenticity of the A, B and C proteins and also indicated that the hnRNP protein profile is much more complex and includes proteins of higher molecular mass. The limitations of the photo-chemical cross-linking are that the cross-linked proteins are no longer useful for biochemical studies and that proteins which bind RNA, but do not cross-link to it efficiently, could be lost. Nevertheless, because of the specificity of this method, we have used UV-cross-linked hnRNPs as a source of genuine hnRNP proteins for immunization of mice to produce monoclonal antibodies to these proteins in order to obtain highly specific probes for the study of individual hnRNP proteins and of intact hnRNP particles.

This strategy (Fig. 1) has now been used successfully for several different cell types including human and yeast and for adenovirus-infected cells. It is a general approach for the identification and production of monoclonal antibodies to nucleic acid binding proteins in vivo. One of the most important applications of the monoclonal antibodies to hnRNP proteins was the immunopurification of hnRNP particles. The method involves a brief incubation of antibodies bound to agarose beads with nuclear lysate (nucleoplasm) and rapid washing of the beads. The procedure is efficient, specific, rapid and mild. It yields pure hnRNP particles and does not require either nuclease degradation or labeling of the hnRNA.

Detailed analysis of the protein composition of immunopurified hnRNP particles from human HeLa cells by two-dimensional gel electrophoresis reveals an assortment of at least 24 polypeptides in the molecular mass range of 34-120 kDa. Some of the complexity seen in this two-dimensional gel may be due to post-translational modification of a smaller number of proteins, but most of the proteins which have an assigned letter are distinct (by immunological and other criteria). The previously described, abundant 30-40 kDa proteins, A, B and C, are a subset of these polypeptides.

The organization of proteins in hnRNP particles
Little is known about the structure of hnRNP particles other than that the bulk of the proteins are organized into monoparticles—complexes that after limited cleavage of the hnRNA with nuclease sediment at 30S-40S and appear by electron microscopy as globular particles of about 200Å. The composition of all monoparticles is presumed to be the same but more direct evidence for this is needed. Most of the proteins shown in Fig. 2 remain together as a complex after digestion with nuclease that generates monoparticles (S. Piñol-Roma and G. Dreyfuss, unpublished). The size of RNA recovered with monoparticles is in the range of 125 to 800 nucleotides. The integrity of the monoparticles is dependent on RNA in that, when the RNA is further degraded, monoparticles dissociate. The prevailing model of the overall structure of hnRNP particles is that of a beads-on-a-string motif with monoparticles forming the beads and the hnRNA itself the string. The simplest interpretation of the pattern of nuclease lability of the hnRNA in hnRNP particles is that the inter-monoparticle (linker) hnRNA is hypersensitive because it is not covered with much protein. Since the monoparticle-associated hnRNA is also RNase sensitive (although less than the linker), it is likely to occupy an exposed position. The 5' cap and the 3' poly(A) tails, when present, are associated with other (non-monoparticle) specific proteins.

Two major questions about the structure of hnRNP particles arise: (1) what is the arrangement of the proteins in monoparticles? and (2) where are monoparticles positioned on any particular pre-mRNA? Related to these questions are the issues of how hnRNP particles assemble and how the packaging of the pre-mRNA into a specific hnRNP structure is related to the processing of pre-mRNA into mRNA. The clarification of the picture of the protein composition of hnRNP monoparticles and the availability of antibody probes to many of these proteins should facilitate the investigation of the arrangement of proteins in monoparticles.

Arrangement of monoparticles?
It is believed (but cannot be stated with certainty) that the positioning of monoparticles on the pre-mRNA is specific and unique for each pre-mRNA. This view is derived primarily from electron micrographs which show protein particles (of similar dimensions to those of monoparticles isolated on sucrose gradients) positioned uniquely on specific transcripts. The inherent difficulty in interpreting these studies is that it cannot be ascertained that the observed particles are indeed hnRNP monoparticles. In fact, monoparticles would not nor-

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**Fig. 2. Two-dimensional gel electrophoresis of hnRNP particles immunopurified with a monoclonal antibody, 4F4, to the C proteins. The hnRNP particles were immunopurified from the nucleoplasm of [35S]methionine-labelled HeLa cells. The proteins were separated by non-equilibrium pH gel electrophoresis in the first dimension and by SDS-PAGE in the second dimension and visualized by fluorography.**
nally be expected to survive the treatment involved in the preparation of the samples for such electron microscopy.

Nevertheless, if a specific arrangement of monoparticles exists, drastically different mechanisms could be envisioned which would give rise to it. The hnRNP proteins could have RNA sequence-dependent binding specificities, although it is widely held that they do not. On the other hand, specific monoparticle positioning could also be achieved either by a processive assembly of monoparticles or by the direction of other factors which do recognize specific RNA sequences, such as small nuclear (sn)RNPs. Knowledge of the RNA-binding properties of the hnRNP proteins and of the assembly process of hnRNP particles is crucial for understanding the structure of hnRNP particles and its relationship to mRNA formation.

The function of hnRNP proteins in mRNA biogenesis

An obvious function for hnRNP proteins is in the packaging of hnRNA. The need for proteins to compact hnRNAs in the nucleus can be understood in terms similar to those for the need to package DNA in chromatin. The packaging must be done, however, in such a way that the pre-mRNA is folded into a structure that can be spliced. This includes the requirement that pre-mRNA is also accessible for interaction with snRNPs. With the development of in vitro cell-free systems which faithfully splice mRNA precursors and with the availability of specific antibodies to proteins of the hnRNP complexes, it has become possible to address the long-standing issue of the involvement of the hnRNP proteins and of the hnRNP complex in pre-mRNA splicing.

The effect on pre-mRNA splicing of several monoclonal antibodies to hnRNPs proteins was investigated. It was found that a monoclonal antibody to the C proteins, two of the major proteins of hnRNP particles (see Fig. 2), inhibits the splicing in vitro of a mRNA precursor (pre-mRNA). It was also found that the splicing complex (spliceosome) contains C proteins and that the splicing complex can be immunoprecipitated with this antibody. Furthermore, immunodepletion of C proteins from the nuclear extract abolishes its capacity to splice RNA and to form spliceosomes. Thus, these experiments suggest that at least two hnRNP proteins, in addition to snRNPs (see Refs. 19 and 20 for reviews), are important for mRNA splicing. A model depicting the overall structure of hnRNP particles and their involvement in mRNA splicing is presented in Fig. 3. Although the specific function of these proteins in the splicing reaction is not yet known, it appears that the antibodies to hnRNP proteins will be valuable tools for studying the splicing complex and the function of hnRNP proteins in mRNA biogenesis.

The transition from hnRNP to mRNPs and nucleo-cytoplasmic transport

Cytoplasmic mRNAs are also associated with proteins (mRNP proteins) although with much less protein than are hnRNAs. The proteins are likely to be involved in modulating the translation and stability of the mRNA and in its cellular localization. In spite of much effort, the picture of the mRNP protein constituents and their arrangement on the mRNA is less well developed than that for the nuclear hnRNP counterparts, but it is clear that the mRNP proteins are different from hnRNP proteins. All of the hnRNP proteins for which antibodies are available (so far) are confined to the nucleus as determined by

![Diagram](https://example.com/diagram.png)

Fig. 3. Schematic presentation of a generalized model of hnRNP particle structure and its involvement in pre-mRNA splicing. For simplicity the spliceosome is illustrated in this scheme as a combination of the snRNPs and hnRNP particles. Not all of the hnRNP proteins, which comprise the bulk of hnRNP particles as seen in Fig. 2, are necessarily in the spliceosome. The double set of arrows for snRNP addition simply illustrate that snRNPs may be associated before, and/or after, addition of hnRNP proteins. E, exon; IVS, intervening sequence (intron); IVS* intron in lariat form; snRNPs, small nuclear ribonucleoprotein particles; PABP, poly(A) binding protein.
immunofluorescence microscopy. These include the A and B proteins\(^2\), the C proteins, the 120 kDa protein\(^2\), the 100 kDa polypyrimidine tract binding proteins, the 70 kDa nuclear matrix proteins, and the UP\(_2\) proteins. The protein sequence of the hnRNPAI, the snRNPAI, the nucleolin, and the UP\(_2\) proteins is found in Ref. 24.

Structure of RNP proteins and the RNP consensus sequence

Once the sequence of one RNP protein became available it was possible to examine whether there is any common sequence motif to RNP proteins. The first two RNP proteins for which amino acid sequence was obtained were the yeast mRNA PABP\(^{12,23}\) and the mammalian A1 hnRNPAI protein\(^{25,26}\). The amino-terminal portion of the PABP contains four similar domains of about 100 amino acids each. The A1 protein contains two similar domains of about 100 amino acids each. Approximately in the middle of these repeating domains is a segment of eight amino acids which is the most strikingly conserved sequence between these domains.

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**TABLE**

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<th>PROTEIN</th>
<th>DOMAIN</th>
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<tr>
<td>PABP YEAST</td>
<td>ESGSVNESSASA YVGDL EPSESEALH YDI FPI VSXV SSY RCVODA YKS DVS MYNEAEKRK LKLYR MNGW YSLR</td>
</tr>
<tr>
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<tr>
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</tr>
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</table>

**CONSENSUS**

| LEU PHE VAL | ILE TYR | ILE PHE VAL LEU VAL THR ASN |
| LEU PHE VAL | ILE TYR | ILE PHE VAL LEU VAL THR ASN |
| LYS ARG | GLY PHE | GLY PHEALA TYR |
| VAL X | PHE | TYP |

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**Fig. 4.** The 93 amino acid RNA binding domains in hnRNPA, mRNPA, snRNPA, and pre-ribosomal RNA-binding proteins. The cytoplasmic mRNPA poly(A) binding protein (PABP) from human\(^2\) and yeast\(^{21,22}\), and the nuclear hnRNPAI proteins from rat and Drosophila\(^{26}\) are illustrated in pairwise comparisons with the vertical lines between amino acids (single letter code) indicating matching pairs. Four of these domains exist in the case of the PABP and two for A1. Also shown are the hnRNPAI proteins C1/C2, the 70 kDa protein from the U1 snRNPA, and the three domains of the nucleolar pre-ribosomal RNA-binding protein, nucleolin.\(^2\) Two regions of extreme amino acid similarity are highlighted by shading. The boxed region, RNP1, corresponds to the RNP consensus sequence, a stretch of eight amino acids described previously\(^{12,24}\), which is the most highly conserved region in the 93 amino acid domains. The striped tilt indicates RNP2 which is the next most conserved region. These regions were assigned by the criteria of having four or fewer different amino acids at that position — other positions meeting this restriction are indicated by an asterisk. Sequences were aligned first by pair-wise comparisons and then visually by including gaps indicated by dots within a sequence. Only a partial sequence is available for the E(UP2) proteins. The citation for the original references for the sequences of the hnRNPAI, the snRNPAI U1 70 kDa, and the UP2 proteins, is found in Ref. 24.
acids segments are RNA-binding domains is based on the following considerations: (1) this feature is common to all of the RNA-binding proteins sequenced; (2) UP1, a 24 kDa amino-terminal portion of A1, which is comprised only of the two 100 amino acid repeats, binds RNA. Similar findings have been made for the C-proteins and PABP (E. Mortenson and G. Dreyfuss, unpublished). Since the 100 amino acid repeats are the RNA-binding domains of the proteins, and since the RNP consensus peptides (RNP1 and RNP2) are the most highly conserved section of these larger domains, it seems likely that they represent important common structures necessary for the RNA-binding domain. It is not yet clear, based on recent experiments for the yeast PABP, whether RNP1 is essential for specific high-affinity RNA binding. Furthermore, the high degree of conservation within the domain suggests that all the proteins that contain it evolved from a common ancestral gene.

In a pairwise comparison of the PABP between yeast and human and those of A1 between rat and Drosophila we have noticed that each domain (indicated as I, II, etc., from the amino terminus of the protein) is significantly more similar to the corresponding domain in a divergent species than it is to any other domain in the same protein (within the same organism). This indicates that each domain has been conserved independently of the others and therefore suggests that the domains in the same protein are not simply redundant repeats but rather serve a different function, albeit probably only subtly so, from the others.

Conclusions and perspectives

Several important advances made over the past several years have led to considerable progress in the knowledge and understanding of hnRNP particles and hnrNP proteins. Thus, whereas several years ago the unambiguous identification of RNA-binding proteins posed considerable difficulty, it may now be possible to classify new proteins as RNP proteins on the basis of the RNP identifier sequence. However, much remains to be investigated about the structure, assembly and disassembly of hnrNP particles, about the detailed mechanism of the participation of hnrNP proteins in mRNA processing and transport and about the structure of the hnrNP proteins and how they interact with hnrNA. The availability of specific antibody and DNA probes as well as the in vitro systems for transcription, RNA splicing and polyadenylation should make additional progress possible.

Acknowledgements

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References


Fig. 5. The percentage similarity of amino acids within RNA-binding domains of RNP proteins. The individual RNA-binding domains of the human and yeast PABP and the rat and Drosophila hnrNP A1 proteins are contrasted with self comparisons (yeast to yeast PABP and rat to rat A1). The percentages indicate amino acid identities between individual domains using pairwise comparisons as shown in Fig. 4. The four RNA binding domains of the PABP and the proline-rich carboxyl terminus (hexagon), and the two domains of the A1 protein and its glycine-rich carboxyl terminus (oval) are also illustrated.
EFTu provides an internal kinetic standard for translational accuracy

Robert C. Thompson

During polypeptide chain elongation the ribosome interacts with aminoacyl-transfer RNA (aa-tRNA) and with elongation factor (EFTu). The rate constants characterizing the ribosome's interactions with EFTu are independent of whether the aa-tRNA is cognate or noncognate. These rate constants act as an internal kinetic standard to measure the kinetics of the ribosome's interaction with the aa-tRNA, and thereby help determine whether the aa-tRNA is cognate or noncognate.

Translation of mRNA into protein is accurate primarily because in response to a particular codon, the ribosome binds one aa-tRNA (cognate) but rejects others (noncognate). Cognate and noncognate ribosome-mRNA-aa-tRNA complexes differ in the extent of base pairing between the codon and anticodon. Although the structural differences between cognate and noncognate codon-anticodon pairs are not yet well understood, our knowledge of the way in which these differences influence the selection of aa-tRNA by the ribosome has advanced recently. A knowledge of the mechanism of this selection process turns out to be essential for understanding translational accuracy because the fidelity of translation of any given codon is not a constant. It can be modulated by a wide variety of genetic and environmental variables that include ribosomal protein and elongation factor mutations, codon context, ppGpp levels, temperature, and polycation concentrations.

Translational accuracy involves two recognition steps

Figure 1 illustrates the minimal mechanism that has been established for the process by which aa-tRNAs are incorporated into nascent protein. As originally proposed on theoretical grounds by Hopfield and by Ninio, ribosomes discriminate against noncognate aa-tRNAs in two distinct steps. In the first of these, known as initial recognition, a ternary complex (TC) of the aa-tRNA, EFTu and GTP binds to the ribosome, and either dissociates (k3) or undergoes GTP hydrolysis (k4). Following acceptance of the ternary complex through GTP hydrolysis, the aa-tRNA is proofread and either is incorporated into the nascent protein chain (k5), or dissociates from the ribosome (k6). Experimental evidence for the existence of this double screen against noncognate aa-tRNAs was first obtained by studying the reaction between poly(U)-programmed ribosomes and a ternary complex of Leu-tRNA2, which is nearly cognate and makes two out of three correct base-pairs with the mRNA. A significant fraction of this ternary complex passes the first discrimination test leading to the hydrolysis of GTP, but most of the aa-tRNA is then rejected (k5) rather than being incorporated into peptide (k6) (Refs 5 and 6). More recent work in a full protein synthesis system also indicates the presence of a proofreading process in aa-tRNA selection by ribosomes.

Translational accuracy requires an internal kinetic standard

Although the two-step nature of the selection process was established by relatively simple experiments, a more complete understanding of the physicochemical basis for discriminating against noncognate aa-tRNAs requires a knowledge of the elementary rate constants k1, k2, k3, k4, k5 and k6. Experiments to determine these values have shown that k1 and k2 are the rate constants that differ most between cognate and non-cognate aa-tRNAs. These experiments also provided a clue to the existence of an internal kinetic standard that enables the ribosome to determine whether an aa-tRNA is cognate or noncognate and prevents the reaction with noncognate aa-tRNA proceeding to the point that an error becomes irreversible. In retrospect it is easy to see the need for some internal standard for accuracy because the ribosome must decide whether an aa-tRNA is cognate without weighing, individually, the relative merits of the 60 or so tRNA complexes in, for example, the E. coli cell.

The most important property of an internal standard is that it be independent of the quantity being measured, in this case the cognate or noncognate nature of the aa-tRNA. Considering first the initial recognition of the aa-tRNA, EFTu and GTP binds to the ribosome, and either dissociates (k3) or undergoes GTP hydrolysis (k4). Following acceptance of the ternary complex through GTP hydrolysis, the aa-tRNA is proofread and either is incorporated into the nascent protein chain (k5), or dissociates from the ribosome (k6). Experimental evidence for the existence of this double screen against noncognate aa-tRNAs was first obtained by studying the reaction between poly(U)-programmed ribosomes and a ternary complex of Leu-tRNA2, which is nearly cognate and makes two out of three correct base-pairs with the mRNA. A significant fraction of this ternary complex passes the first discrimination test leading to the hydrolysis of GTP, but most of the aa-tRNA is then rejected (k5) rather than being incorporated into peptide (k6) (Refs 5 and 6). More recent work in a full protein synthesis system also indicates the presence of a proofreading process in aa-tRNA selection by ribosomes.7

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