SPECIFICITY IN THE INTERACTION OF hnRNA AND mRNA WITH PROTEINS AS REVEALED BY IN VIVO CROSS LINKING

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1. Introduction

In eukaryotic cells mRNA as well as hnRNA are found to be associated with proteins in the form of m-ribonucleoprotein and hn-ribonucleoprotein particles, respectively [1–4]. The mRNA and hnRNA associated proteins may play an important role in the processing of hnRNA to mRNA, in RNA transport and in the regulation of mRNA translation. Many reports have appeared concerning the protein composition of these RNP particles [1–9]. It is now generally agreed upon that hnRNP particles contain a group of so-called core proteins in the molecular weight range of 30 000 to 44 000 [2,3,7,8] and that mRNA is associated with at least two proteins of approx. 50 000 and 75 000 M_r [5,6,9]. In these studies mRNP and hnRNP particles were purified from cytoplasmic and nuclear extracts by sedimentation or density-gradient centrifugation or by oligo(dT)-cellulose chromatography before analysis of their protein content. These methods of isolation have the generally acknowledged disadvantage that non-specific binding of proteins to the RNA during cell fractionation may occur. Furthermore, no definite information is obtained about the proteins that directly interact with the RNA, as some proteins may be associated with the RNP particles for example via protein–protein interactions.

To identify the proteins that are tightly associated with RNA we have used irradiation with high doses of ultraviolet light (254 nm) as a method for RNA–protein cross-linking [10–12]. To be certain that only proteins interacting in vivo with RNA were covalently linked, we performed the irradiation on intact cells. Only proteins closely associated with RNA can be covalently linked by ultraviolet irradiation (for references see [10]). Furthermore isolation of the covalent RNA–protein complexes can be performed under conditions that exclude co-purification of non-specifically associated proteins. Covalently linked polyadenylated mRNA–protein and hnRNA–protein complexes can be purified by oligo(dT)-cellulose chromatography and the protein part of the complexes analysed after extensive nuclease treatment. If the cells were prelabeled with RNA precursors, the cross-linked proteins can be detected after gel electrophoresis by fluorography, owing to the residual radioactive nucleotides covalently linked to them.

In this study we have used this method to compare the proteins cross-linked to mRNA with those cross-linked to hnRNA. By using various radioactive RNA precursors, it was found that distinct nucleotides are involved in the cross-linking of RNA to some of the proteins.

2. Materials and methods

HeLa S3 cells were grown in suspension as described before [12]. Labeling of RNA was performed in cell suspensions with densities up to 2×10^6 cells ml^{-1} with 10 μCi ml^{-1} [2,5',-8-^{3}H]adenosine (42 Ci $mmol^{-1}$), [5-^{3}H]cytidine (31 Ci $mmol^{-1}$), [8-^{3}H]-guanosine (5 Ci $mmol^{-1}$) or [5,6-^{3}H]uridine (40 Ci $mmol^{-1}$) (all obtained from the Radiochemical Center, Amersham) for 4 h at 37°C. After labeling, the cells were harvested on frozen saline and irradiated for 3 min with ultraviolet light as described [10,12]. The radiation dose at 254 nm received by the sample was 8000 J m^{-2} (10^{22} quanta m^{-2}) per minute [10]. Cell fractionation was performed as described earlier [10,12]. Cytoplasmic and nuclear RNP containing fractions were made 1% in SDS and heated for 2 min at 90°C to dissociate all non-covalent RNA–protein

complexes. Polyadenylated RNA and covalent RNA–protein complexes were isolated from these solutions by oligo(dT)-cellulose chromaography as described [10,12]. The eluates were treated with RNase A (25 μg ml^{-1}, Sigma Chemical Co.) and micrococcal nuclease (400 U ml^{-1}, P-L Biochemicals Inc.) in the presence of 2 mM Ca^{2+} for 1 h at 37°C. The proteins from the resulting mixture were analyzed on SDS–polyacrylamide gels, as described [10,12]. Control experiments verifying the specificity of the in vivo cross-linking method and subsequent selection procedure via oligo(dT)-cellulose have also been described [10–12].

3. Results and discussion

In previous studies we have shown that irradiation of intact cells with high doses of ultraviolet light induces covalent cross-links between mRNA or hnRNA and proteins [10–12]. Under our experimental conditions, 50–80% of the cellular mRNA and hnRNA are found to be cross-linked to protein after 3 min of irradiation (corresponding to a radiation dose of 24 000 J m^{-2}). Although as a result of the irradiation proteins become covalently attached to the RNA and most probably also to the 3′ poly A region, the covalent RNA–protein complexes bind efficiently to oligo(dT)-cellulose in the presence of SDS and 0.5 M NaCl and can be eluted with low salt buffer. The retention of the RNA–protein complexes is completely dependent on the integrity of the RNA and is not due to direct binding of the proteins to the column [10–12].

In the type of experiment discussed here, cellular RNA was labeled by incubation of separate portions of cells with [^{3}H]adenosine, [^{3}H]cytidine, [^{3}H]guanosine or [^{3}H]uridine. The cells were then harvested, irradiated for 3 min and fractionated. The poly A(+) mRNA–protein and hnRNA–protein complexes were purified from cytoplasmic and nuclear fractions by oligo(dT)-cellulose chromatography and treated with nucleases (see Materials and methods) to degrade the RNA part of the complex as completely as possible. From the percentage of RNA counts that remain associated with the proteins after nuclease treatment [10,12] and the average size of the mRNA and hnRNA molecules, it can be calculated that 1–3 nucleotides remain associated per covalently linked protein after nuclease digestion. In this way the proteins are marked by the residual [^{3}H]nucleotides, without significantly influencing their migration during SDS–gel electrophoresis. By labeling the cellular RNA separately with ^{3}H-labeled adenosine, cytidine, guanosine or uridine we investigated if the patterns of the proteins cross-linked to mRNA and hnRNA showed differences depending on the nucleoside used. Fig.1 shows a typical result of such an experiment.

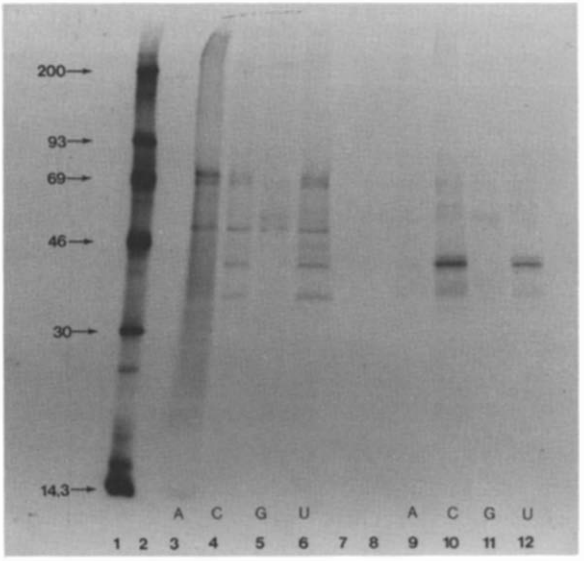

Fig.1. Analysis of proteins cross-linked to poly A(+) mRNA and poly A(+) hnRNA. 6 × 10^8 HeLa cells were divided in four equal portions and labeled separately with [^{3}H]adenosine, [^{3}H]cytidine, [^{3}H]guanosine or [^{3}H]uridine, as described in Materials and methods. The cells were irradiated with ultraviolet light for 3 min. Poly A containing mRNA and mRNA–protein complexes as well as poly A containing hnRNA and hnRNA–protein complexes were isolated from these cells (see Materials and methods). After extensive nuclease treatment [10,12] the RNA-linked proteins were analyzed on 10–18% polyacrylamide gels. These proteins were detected by fluorography owing to the ^{3}H-labeled nucleotides still attached to them. Lane 1: ^{14}C-labeled marker proteins. M_r values: 200 000; 93 000; 69 000; 46 000; 30 000; 14 300. (Lane 2, empty.) Lane 3: proteins cross-linked to [^{3}H]A labeled poly A(+) mRNA; lane 4: proteins cross-linked to [^{3}H]C labeled poly A(+) mRNA; lane 5: proteins cross-linked to [^{3}H]G labeled poly A(+) mRNA; lane 6: proteins cross-linked to [^{3}H]U labeled poly A(+) mRNA; lane 7: proteins covalently attached to poly A(+) mRNA from unirradiated cells labeled with ^{3}H-labeled A,C,G and U. lane 8: proteins covalently attached to poly A(+) hnRNA from unirradiated cells labeled with ^{3}H-labeled A,C,G and U. lane 9: proteins cross-linked to poly A(+) hnRNA labeled with [^{3}H]A; lane 10: proteins cross-linked to poly A(+) hnRNA labeled with [^{3}H]C; lane 11: proteins cross-linked to poly A(+) hnRNA labeled with [^{3}H]G; lane 12: proteins cross-linked to poly A(+) hnRNA labeled with [^{3}H]U.

Lanes 3–6 show the proteins cross-linked to polyadenylated mRNA, that are labeled with ^{3}H-labeled adenosine, cytidine, guanosine and uridine, respectively. Four major proteins (M_r 73 000, 69 000, 52 000 and 41 500) are cross-linked. The lanes 9–12 show the proteins cross-linked to polyadenylated hnRNA from the same portions of cells. In agreement with our earlier results, two proteins of M_r 41 500 and 43 000 are found to be cross-linked. Surprisingly these two proteins were only detected when the cells were labeled with [^{3}H]cytidine or [^{3}H]uridine and not when the other tritiated precursors were used. This is not due to poor labeling since the amount of radioactive poly A(+) hnRNA from [^{3}H]adenosine labeled cells was comparable to the amount obtained from [^{3}H]cytidine- or -uridine-labeled cells. Because of the lower specific activity of [^{3}H]guanosine, lower number of counts incorporated into polyadenylated hnRNA were obtained when this precursor was used (about 20% as compared to the [^{3}H]cytidine and -uridine-labeled samples). However, the presence of a specific band at 55 000 M_r (lane 11) does suggest that the lower specific activity is not the proper explanation for the absence of the M_r 41 500 and 43 000 bands. There are two obvious explanations for the observed base specificity in the cross-linking reaction. One is that pyrimidines cross-link more efficiently during irradiation with ultraviolet light than purines and, secondly, that there is some specificity in the RNA sequence to which these hnRNP proteins are associated. The fact that proteins are efficiently cross-linked to mRNA from the same portion of adenosine-labeled cells argues strongly against the first explanation. It thus seems more plausible that there is some kind of specificity in the interaction of hnRNA with the M_r 41 500 and 43 000 proteins that causes these proteins to cross-link only to uridine and cytidine. Possibly the proteins are tightly associated with a U–C-rich sequence in the hnRNA or, alternatively, only some pyrimidines have the correct spatial orientation for covalent bonding to proteins.

Base specificity is, to a lesser extent, also found in the case of mRNA–protein cross-linking. Fig.1, lane 3 shows that prelabeling with [^{3}H]adenosine reveals a strongly labeled protein band of 73 000 M_r. When the intensities of both the M_r 52 000 and 73 000 bands in the lanes 3,4,5 and 6 compared it is obvious that the M_r 73 000 protein is most strongly labeled when [^{3}H]adenosine was used as precursor. This could be expected since it is known that a protein of similar molecular weight is associated with the 3′ poly A tail of mRNA [9]. The fact that the poly A-binding protein is also found when [^{3}H]uridine or -cytidine were used as precursors suggests that this protein can bind to regions of the mRNA other than poly A. This is possible since it has been found earlier [13] that the poly A-rich part of HeLa cell mRNA, protected against RNase treatment by the poly A-binding protein, contained a surprisingly high content (25%) of UMP residues. Recently, protection of RNA sequences adjacent and also non-adjacent to the poly A region by poly A-binding proteins has been reported [14]. Another possibility is that there is more than one protein of this size associated with mRNA, as has been suggested by Greenberg [15].

We were not able to detect the M_r 73 000 protein on polyadenylated nuclear RNA, although the association of this protein with hnRNA has been reported [16,17]. This can either mean that the presumed nuclear localization of this protein was due to cytoplasmic contamination or that the amount of this protein cross-linked to hnRNA is beyond our detection limit.

Cytidine and uridine labeling of cytoplasmic polyadenylated RNA clearly reveals a protein of M_r 41 500, which comigrates with the major protein band in the corresponding nuclear samples. Although it cannot be excluded that this band represents a genuine mRNP protein, it is probably due to nuclear contamination as the intensity of this band proved to be rather variable in different experiments, as compared to the three other major bands which had constant relative intensities (see also [10,11]). Finally, labeling with [^{3}H]guanosine reveals a M_r 55 000 protein (lane 5), which probably corresponds to the M_r 55 000 protein found associated with hnRNA after guanosine labeling (lane 11). This protein is not detected after labeling the RNA with the other three precursors. Control experiments (lane 7 and 8) show that no proteins were bonded to poly A(+) mRNA or hnRNA when cells had not been irradiated with ultraviolet light.

Our results clearly show that cytoplasmic poly A(+) mRNA is associated with a set of proteins that is different from the proteins associated with poly A(+) hnRNA. Thus during transport of RNA from nucleus to cytoplasm, that is during the transition of hnRNA to mRNA, the set of proteins interacting tightly with the RNA changes. Furthermore we have shown that distinct nucleosides are interacting with

these proteins. This suggests that these proteins bind to specific RNA sequences.

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