Proteins crosslinked to messenger RNA by irradiating polyribosomes with ultraviolet light

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ABSTRACT

A set of proteins crosslinked to L-cell mRNA by irradiating polyribosomes with 254 nm ultraviolet light has been identified. ³⁵S-methionine-labeled crosslinked mRNA-protein complexes were isolated by chromatography on oligo(dT)-cellulose under conditions that prevented non-covalent binding of proteins to RNA and to the column. After enzymatic removal of the RNA the proteins were analyzed in sodium dodecylsulfate/polyacrylamide gels. Six proteins having molecular weights of 98,000, 78,000, 75,000, 68,000, 62,000, and 52,000 were crosslinked to mRNA whether intact polyribosomes or EDTA-released mRNA-protein complexes were irradiated. Digestions with specific RNAases and chromatography on oligo(dT)-cellulose were used to show that a protein of 78,000 daltons was the only one crosslinked to poly(A), and the other proteins were crosslinked to sequences other than poly(A). However, a 78,000 dalton protein was also crosslinked to a sequence other than poly(A).

INTRODUCTION

There is widespread agreement that mRNA-protein complexes released from mammalian and avian polyribosomes by treating them with EDTA or with high salt and puromycin contain two proteins having molecular weights of approximately 78,000 and 52,000 (reviewed in references 1,2). Also, as first shown by Blobel (3) the 78K protein is associated with poly(A). Aside from these two proteins, however, there is little agreement among different laboratories on the numbers or sizes of mRNA-associated proteins, and their functions remain obscure. This situation is due in large part to a lack of satisfactory criteria for the identification of mRNA-associated proteins and to a lack of suitable methodology for working with them.

The problems are twofold. Firstly, it is necessary to isolate mRNP without either dissociating it or allowing contamination by extraneous proteins to occur, and secondly, it is necessary to separate it from ribosomes. The first problem is especially troublesome, and it is usually not possible to be sure that isolated mRNP is either native or free of extraneous

proteins. Many workers have relied on stability of mRNA-protein complexes in high salt, e.g., 0.5 M KCl, as a criterion for identifying mRNA-associated proteins (1,2). Although it is likely that proteins remaining associated with mRNA under these conditions are true mRNA-associated proteins, it is uncertain to what extent genuine mRNA-associated proteins are removed from mRNP by this treatment. One important technique is the isolation of poly(A)⁺mRNP by chromatography on oligo(dT)-cellulose (4). This procedure is useful for separating mRNP from ribosomes. However, it is usually necessary to elute oligo(dT)-cellulose-bound mRNP by using agents such as formamide that disrupt RNA-protein complexes. For this reason it is difficult to be sure that proteins were bound to the column because of their association with poly(A)⁺mRNA.

Because of the foregoing considerations it seemed important to develop new criteria for defining and identifying mRNA-associated proteins and new methodology for working with them. One possible approach is to use a RNA-protein crosslinker of such specificity that only proteins truly associated with mRNA can be crosslinked to it. If such a crosslinker were available, then polyribosomes could be isolated under conditions which minimize dissociation of proteins from RNA, i.e., at low ionic strength, and crosslinking could be used to identify mRNA-specific proteins. One crosslinker that has proved effective in investigating various types of protein-nucleic acid interactions is irradiation by UV light. Many striking examples show that irradiation by UV light can covalently join proteins to nucleic acids in a manner that is highly specific with respect to both the amino acid and nucleotide sequences involved in crosslinking (5-14). Apparently, the basis for the sequence specificity of UV-induced crosslinking is the inherent sequence specificity of protein-nucleic acid interactions together with the short effective crosslinking range of UV light. Both pyrimidine and purine bases can participate in crosslinking (15-21). Also, up to eleven different amino acids can be involved (16, 18).

Because of the extensive evidence that UV-induced crosslinking is both sequence-specific and generally applicable it was decided to apply this approach to the study of mRNA-protein interactions. In an earlier paper (22) it was shown that mRNA could be extensively crosslinked to proteins by irradiating isolated polyribosomes with 254 nm light. The mRNA was not degraded or crosslinked to other RNA molecules, and after enzymatic deproteinization it could be analyzed by gel electrophoresis. Histone mRNA was crosslinked to many fewer proteins than most mRNAs, and 4S RNA was not

appreciably crosslinked to protein. In the present paper the proteins crosslinked to mRNA are analyzed by methods analogous to those previously used for mRNA analysis. It is shown that proteins crosslinked to poly(A)⁺mRNA can be isolated by chromatography on oligo(dT)-cellulose under conditions that dissociate non-covalent RNA-protein complexes, and a set of proteins crosslinked to poly(A)⁺mRNA has been identified. A protein of 78,000 daltons was the only one crosslinked to poly(A). However, a protein of the same size could be crosslinked to a non-poly(A)-region of poly(A)⁺mRNA. Therefore, the 78K protein (or proteins) is not simply a poly(A) binding protein.

MATERIALS AND METHODS

Cell culture, labeling, and fractionation. Mouse L-cells were suspended in spinner medium containing 1.9 µg/ml of L-methionine, which is 1/8 the usual concentration. The medium contained non-dialyzed 4% calf serum and 4% fetal calf serum. After overnight growth to a density of 3-3.5 x 10⁵ cells/ml they were incubated 30 min with 0.08 µg/ml of actinomycin D, then concentrated eight-fold in the same medium and labeled for 70 min with 10 µCi/ml of ³⁵S-methionine and 10 µCi/ml of ³H-adenosine (obtained from New England Nuclear). In one experiment cells were labeled with ³H-leucine as described previously (22). Polyribosomes were isolated as described previously (23). Briefly, cells were lysed in isotonic KCl buffer containing Triton-X-100, and polyribosomes were purified by centrifuging through sucrose gradients containing 0.01M NaCl, 0.002 M MgSO₄, and 0.01 M Tris-HCl (pH 7.6).

UV-irradiation and oligo(dT)-cellulose chromatography. Polyribosomes were irradiated as described (22). For chromatography on oligo(dT)-cellulose the polyribosomes were ethanol-precipitated. Dried pellets containing ≤ 10 A₂₆₀ units of polyribosomes were dissolved in 0.45 ml of 0.5% sodium dodecyl-sulfate, 0.01M Tris-HCl (pH 7.6), and 0.001 M EDTA. In most experiments the samples also contained 1% 2-mercaptoethanol. The bottoms of the tubes were scraped to assure complete resuspension, and the samples were made 0.5 M in NaCl by addition of 5 M NaCl. The samples were heated at 60°C for three min, then cooled to room temperature and applied to a column containing 0.1 g of oligo(dT)-cellulose T₃ (Collaborative Research) equilibrated with the sample buffer. Unbound material was eluted with 2.5 ml of the sample buffer, then bound material was eluted by washing with 3 ml of elution buffer which consisted of sample buffer lacking NaCl. The eluate was made 0.5 M in NaCl, heated again to 60°C, and recycled through the column. For

isolation of poly(A) and protein crosslinked to it the poly(A)⁺mRNA and associated proteins were precipitated with ethanol, and the dried precipitate was dissolved in 0.5 ml of 0.5 M NaCl, 0.01 M Tris·HCl (pH 7.6), 0.001 M EDTA, 0.5% sodium lauroyl sarcosinate, 1% 2-mercaptoethanol, digested for one hour at 37°C with 5 µg of RNAase A and 50 units RNAase T₁, heated to 60°C, cooled, and applied to the oligo(dT)-cellulose column. The column was washed and eluted as described for undigested material.

Further analysis of samples. In some cases fractions from the oligo(dT)-cellulose column were analyzed further to separate free protein from cross-linked RNA-protein complexes. Samples were fractionated on the basis of buoyant density by centrifugation in Cs₂SO₄-urea gradients as described (22). For fractionation on the basis of size samples were layered on linear sucrose gradients made from equal volumes of 15% (w/w) sucrose and 30% (w/w) sucrose. The sucrose was dissolved in 0.5% sodium dodecylsulfate, 0.1 M NaCl, 0.01 M Tris·HCl (pH 7.6), 0.001 M EDTA. 34 ml gradients were centrifuged 17 hours at 20,000 rpm at 22°C in a Beckman SW27 rotor. 10 ml gradients were centrifuged 17 hours at 22,000 rpm at 22°C in a Beckman SW 41 rotor. The gradients were collected through the flow cell of a recording spectrophotometer or UV monitor in order to detect the position of rRNA peaks, and about 30 equal fractions were collected.

RNAase digestion and gel electrophoresis. Samples from the oligo(dT)-column were ethanol-precipitated. The precipitates were pelleted by centrifugation, washed with ethanol, and air-dried at 37°C. In early experiments, e.g., Figure 3, precipitates were dissolved in 0.5 ml of 0.05 M sodium acetate buffer (pH 4.5) containing 0.5% sodium lauroyl sarcosinate and digested for 1-2 hours at 37°C with 20 units of RNAase U₂ (Calbiochem). RNAases A and T₁ were also included if the samples had not been previously digested with these enzymes. The samples were then ethanol-precipitated and dissolved in gel application buffer. In later experiments, e.g., Figures 4,5, samples were dissolved in 0.15 ml of 0.0625 M Tris·HCl (pH 6.8), 0.5% sodium lauroyl sarcosinate, 10% (v/v) glycerol, and 1% 2-mercaptoethanol. The samples were digested at 37°C for one hour with 12.5 units of RNAase T₂ (Sigma Grade VI), then made 2% in sodium dodecylsulfate and 0.001% in bromophenol blue by addition of a 10X stock solution, boiled for two min, and applied directly to the gel. The proteins were analyzed in 10% polyacrylamide slab gels containing sodium dodecylsulfate as described by Laemmli (24). The resolving gel was 8.5 cm in length, and the spacer gel was 1 cm in length. The gels were 1.5 mm thick. Molecular weight markers of ¹⁴C-labeled proteins (ob-

tained from New England Nuclear) were run in each gel in parallel with the ^{35}S -labeled proteins. The markers were phosphorylase A ($M_r = 92,000$), bovine serum albumin ($M_r = 68,000$), ovalbumin ($M_r = 45,000$), carbonic anhydrase ($M_r = 30,000$), and cytochrome C ($M_r = 12,500$). The gels were fluorographed as described by Laskey and Mills (25) using preflashed Kodak XR-5 film.

Scintillation counting. Aliquots of fractions from oligo(dT)-cellulose columns, sucrose gradients, and Cs_2SO_4 gradients were counted in Aquasol (New England Nuclear) or Hydrofluor (National Diagnostics). ^3H counts were corrected for ^{35}S counts spilling into the ^3H channel. It was determined by external standardization that spillover was constant for samples containing varying amounts of sucrose and Cs_2SO_4 .

RESULTS

In order to label mRNA-protein complexes without labeling rRNA or ribosomal structural proteins L-cells were treated with $0.08\text{ }\mu\text{g/ml}$ of actinomycin D (26, 27). They were then doubly labeled with ^{35}S -methionine and ^3H -adenosine. Poly(A) $^+$ mRNA-protein complexes were isolated from UV-irradiated polyribosomes by chromatography on oligo(dT)-cellulose in the presence of sodium dodecylsulfate. This procedure differs from previous uses of oligo(dT)-cellulose chromatography for isolation of mRNP in that only proteins covalently joined to poly(A) $^+$ mRNA are expected to bind to the column. That binding of protein to the column is UV dose-dependent is shown in Figure 1. The fraction of labeled protein bound increased with UV dose up to 10% at a dose of 10^5 ergs/mm^2 . One percent of the labeled protein was bound in the absence of irradiation. The fraction of labeled RNA bound appeared to be independent of UV dose, and it remained constant at about 60%. In the experiment shown chromatography was done in the absence of 2-mercaptoethanol, and the samples were not heated prior to chromatography. In subsequent experiments in which 2-mercaptoethanol was present, and the samples were heated to 60°C prior to chromatography, a lower level of protein binding was obtained, typically, 4-5% at doses of $10^5 - 2 \times 10^5\text{ ergs/mm}^2$ and $\leq 0.5\%$ in the absence of irradiation. Although it has not been ruled out that some crosslinks are reversed by heating, the more rigorous conditions have been used routinely so as to select for the most stable complexes and to minimize non-specific binding of proteins to RNA and to the column. Two cycles of chromatography were routinely used to isolate the crosslinked complexes. In the second cycle typically 95% of

Figure 1. UV dose dependence of crosslinking. Polyribosomes from cells doubly labeled with ^{35}S -methionine and ^3H -adenosine were irradiated to the indicated incident doses as described (22). After irradiation the polyribosomes were precipitated with ethanol. The precipitates were spun down, dried, dissolved in application buffer, and chromatographed on oligo(dT)-cellulose as described in "Materials and Methods". The amount of radioactivity bound after two cycles of chromatography is shown. The horizontal line drawn through the open circles and the vertical bar represent the mean and standard deviation of the ^3H -radioactivity bound (59.6 ± 3.7). x—x ^{35}S . o—o ^3H .

the ^3H cpm and 70-80% of the ^{35}S cpm bound in the first cycle were rebound.

In order to confirm that most of the protein bound to oligo(dT)-cellulose as a result of irradiation was crosslinked to RNA the eluted material was centrifuged in sucrose gradients containing sodium dodecyl-sulfate. These gradients presumably fractionate RNA-protein complexes on the basis of size. Figure 2a shows that the proteins remain near the top of the gradient when unirradiated polyribosomes are centrifuged. mRNA sediments in a broad peak centered at 18S. However, the proteins and mRNA bound to oligo(dT)-cellulose after irradiation sediment very heterogeneously, and there is no peak of protein radioactivity at the top of the gradient (Figure 2b). On the basis of sedimentation behavior at least 80% of the protein bound to the column was crosslinked to RNA. If the RNA-protein complexes are treated with RNAase prior to centrifugation most of the labeled proteins once again remain near the top of the gradient (Figure 2c), as expected if they had been crosslinked to RNA before digestion.

In order to identify the proteins crosslinked to poly(A)⁺ mRNA the ^{35}S -

Figure 2. Demonstration of protein cross-linking to poly(A)⁺mRNA by sedimentation in sucrose gradients containing sodium dodecyl sulfate. Unirradiated polyribosomes or RNA and protein bound to oligo(dT)-cellulose after irradiation of polyribosomes were sedimented as described in "Materials and Methods". The positions of 28S and 18S rRNA markers were determined by UV scanning of gradients containing unlabeled rRNA (A,B), or by counting internal markers of ¹⁴C rRNA (C). The gradients in A and B were centrifuged in the SW 27 rotor. The gradients in C were centrifuged in the SW 41 rotor. A) Unirradiated polyribosomes doubly labeled with ³⁵S-methionine and ³H-adenosine. B) ³H-adenosine-labeled RNA and ³⁵S-methionine-labeled protein bound to oligo(dT)-cellulose from irradiated doubly labeled polyribosomes. C) ³H-leucine-labeled protein bound to oligo(dT)-cellulose from irradiated polyribosomes. x—x Undigested control. o—o Digested with RNAase T2.

labeled RNA-protein complexes bound to oligo(dT)-cellulose after irradiation were eluted and digested with nucleases that hydrolyze nearly all phosphodiester bonds in RNA, *i.e.*, RNases A, T₁, and U₂, or RNAse T₂ alone. The proteins were then analyzed in sodium dodecylsulfate/polyacrylamide gels. Figure 3c shows that there were six bands detectable in the gel of proteins crosslinked to poly(A)⁺mRNA. They had apparent molecular weights of 98,000, 78,000, 75,000, 68,000, 62,000 and 52,000. The labeled proteins of unirradiated polyribosomes contained a larger number of bands (Figure 3b). However, there were major bands corresponding in electrophoretic mobility to the proteins crosslinked to poly(A)⁺mRNA. There was considerable radioactivity remaining near the origin of the gel after irradiation (Figure 3c). This radioactivity presumably resulted from protein-protein crosslinking, which is known to occur to some extent at 254 nm (28). A more extensive nuclease treatment did not increase the amount of radioactivity entering the gel (for example, see Figure 5c, d). In the experiment shown in Figure 3c the irradiated polyribosomes were not heated or treated with 2-mercapto-ethanol prior to chromatography. However, the pattern of bands is the same

Figure 3. Analysis of proteins crosslinked to poly(A)⁺mRNA. Doubly labeled polyribosomes were irradiated to a dose of 10^5 ergs/mm² and chromatographed on oligo (dT)-cellulose. The material bound after two cycles of chromatograph was eluted, ethanol-precipitated, digested with RNAases A, T₁, and U₂, and analysed in a polyacrylamide gel containing sodium dodecylsulfate. A sample of total labeled proteins from unirradiated polyribosomes was analyzed similarly. The numbers at the left and right of the figure correspond to the molecular weights in thousands of daltons of the standards and the proteins crosslinked to poly(A)⁺mRNA respectively. A) Standards. B) unirradiated polyribosomes. C) Proteins crosslinked to poly(A)⁺mRNA.

as that obtained in subsequent experiments in which the more rigorous conditions were used (Figures 4,5). In a number of experiments at UV doses ranging from 1×10^5 to 3×10^5 ergs/mm² the same six bands were always observed. However, there was some loss of band sharpness, and there was increased background radioactivity, as well as increased protein-protein crosslinking at the higher doses. A number of minor bands was reproducibly observed in addition to the major bands indicated. However, it is not known if they were significant.

In the experiments presented thus far intact polyribosomes were irradiated, and the possibility was considered that the proteins crosslinked to mRNA under these conditions are not identical with the proteins of EDTA-released mRNP, which has been investigated by others who did not use crosslinking. Also, the possibility must be considered that UV irradiation alters the structure of polyribosomes in such a way that proteins not in close contact with mRNA in native polyribosomes are brought close enough for crosslinking to occur. Such a possibility is suggested by the work of Gorelic (29). In order to check these possibilities polyribosomes were irradiated in the presence and in the absence of EDTA, which is known to disassemble polyribosomes into ribosomal subunits and mRNA-protein complexes (30, 31). Irradiation does not by itself cause breakdown of polyribosomes or interfere with subsequent disassembly by EDTA (unpublished observations). It was found that treatment with 0.005 M EDTA slightly increased the extent

of protein crosslinking to mRNA (20-40% in two separate experiments). However, when the proteins crosslinked to mRNA in the presence and absence of EDTA were analyzed in sodium dodecylsulfate/ polyacrylamide gels no differences were found (Figure 4), and it is concluded that the set of labeled proteins crosslinked to mRNA is the same whether intact polyribosomes or EDTA-released mRNA-protein complexes are irradiated.

One of the proteins crosslinked to poly(A)⁺mRNA, the 78K protein, was similar in size to the protein found by others to be associated with poly(A) (3, 32-37). Therefore, it was of interest to determine whether or not this protein could be crosslinked to poly(A). For this purpose doubly labeled crosslinked mRNA-protein complexes were isolated by chromatography on oligo(dT)-cellulose, then digested with RNAases A and T₁ in 0.5 M NaCl. Under these conditions poly(A) is preserved, but nearly all other phosphodiester bonds in RNA are hydrolyzed (38). The poly(A) segment and any protein crosslinked to it were then isolated by rechromatography on oligo(dT)-cellulose. About 30% of the ³H-adenosine radioactivity in poly(A)⁺mRNA was rebound to the column after digestion with or without irradiation. This value agrees with a previous estimate for the fraction of mRNA ³H-adenosine radioactivity in poly(A) after a label of similar duration (39), and it is concluded that binding of poly(A) to the column was unaffected by irradiation. About 10% of the ³⁵S-radioactivity in proteins crosslinked to poly(A)⁺mRNA was rebound to oligo(dT)-cellulose after digestion with RNAases

Figure 4. Proteins crosslinked to mRNA of intact polyribosomes and EDTA-released mRNP. ³⁵S-labeled polyribosomes were irradiated to a dose of 2×10^5 ergs/mm² in the presence or absence of 0.005 M EDTA. Poly(A)⁺mRNA-protein complexes were isolated by chromatography on oligo(dT)-cellulose, and the proteins were analyzed in gels after digestion with RNAase T₂. A) EDTA-released mRNP. B) Intact polyribosomes.

A and T₁. In order to analyze the proteins crosslinked to poly(A) and sequences other than poly(A) the bound and unbound fractions were digested with RNAase T₂, a nonspecific RNAase that digests poly(A) (40), and the proteins were analyzed in sodium dodecylsulfate/polyacrylamide gels. In preliminary experiments a single protein of 78,000 daltons was rebound to oligo(dT)-cellulose after digesting the complexes with RNAases A and T₁. This protein was presumably crosslinked to poly(A). The other proteins crosslinked to mRNA, *i.e.*, the 98K, 75K, 68K, 62K, and 52K proteins, were detected only in the non-rebound materials, and they were presumably crosslinked to mRNA sequences other than poly(A). Unexpectedly, however, a 78K band was also prominent among the proteins not rebound to oligo(dT)-cellulose. In fact, there was as much or more radioactivity in 78K protein in the non-rebound fraction as in the rebound fraction.

In order to determine whether the 78K protein in the non-rebound fraction was truly crosslinked to some sequence other than poly(A) an additional RNAase digestion experiment was done. The proteins of the isolated poly(A)⁺mRNA-protein complex, as well as the material rebound to oligo(dT)-cellulose and the material not rebound after digestion of this complex with RNAases A and T₁ were analyzed in gels with or without RNAase T₂ digestion. Figures 5a and 5b show that the proteins of the poly(A)⁺mRNA-protein complex enter the gel after RNAase T₂ digestion, but not in the absence of RNAase T₂ digestion. These results are evidence that the proteins were crosslinked to RNA and that the RNAase T₂ treatment was effective. Figure 5e and 5f show that the 78K protein rebound to oligo(dT)-cellulose after digestion with RNAases A and T₁ enters the gel after RNAase T₂ digestion, but not after digestion with RNAases A and T₁ alone. Figure 5c and 5d show that the proteins not rebound to oligo(dT)-cellulose after digestion with RNAases A and T₁ enter the gel without further nuclease treatment, and that treatment with RNAase T₂ makes no difference in the pattern of bands or in the amount of radioactivity remaining at the origin. These results confirm that the 78K protein was crosslinked to poly(A), and that the 98K, 75K, 68K, 62K, and 52K proteins, and a portion of the 78K protein were crosslinked to sequences other than poly(A).

Proteins crosslinked to RNA not bound to oligo(dT)-cellulose. When the extent of labeled protein crosslinking to RNA not bound to oligo(dT)-cellulose after UV irradiation of polyribosomes was analyzed it was found that at least twice as many cpm of labeled protein were crosslinked to unbound RNA as were crosslinked to bound RNA. Crosslinking was assayed by

Figure 5. Effects of RNAase T₂ digestion on the electrophoretic mobility of proteins rebound or not rebound to oligo(dT)-cellulose after digesting poly(A)⁺mRNA-protein complexes with RNAases A and T₁. ³⁵S-methionine-labeled poly(A)⁺mRNA-protein complexes were isolated by chromatography on oligo(dT)-cellulose from intact polyribosomes irradiated to 2×10^5 ergs/mm². A portion of the complexes was digested with RNAases A and T₁ in 0.5 M NaCl. The total proteins of these complexes and the proteins rebound or not rebound to oligo(dT)-cellulose after digestion with RNAases A and T₁ were analyzed in gels after digestion with RNAase T₂ (A,C,E), or without RNAase T₂ digestion (B,D,F). A, B) Total proteins crosslinked to poly(A)⁺mRNA, 20,000 cpm per gel, nine day exposure. C, D) Proteins not rebound to oligo(dT)-cellulose after digestion with RNAases A and T₁, 20,000 cpm per gel, five day exposure. E, F) Proteins rebound to oligo(dT)-cellulose after digestion with RNAases A and T₁, 2,000 cpm per gel, 16 day exposure.

centrifugation in both sodium dodecylsulfate/sucrose gradients and Cs₂SO₄/urea gradients. The unbound RNA to which the proteins were crosslinked was probably not newly synthesized poly(A)⁺mRNA which failed to bind to oligo(dT)-cellulose, since it was previously shown that newly synthesized L-cell poly(A)⁺mRNA is quantitatively bound (23), and the UV irradiation had little effect on the extent of labeled mRNA binding to the column. However, when the proteins crosslinked to the non-bound RNA were analyzed they were found

to have the same molecular weights as the proteins crosslinked to bound RNA except for the presence of a few additional bands. The nature of the unbound RNA to which the proteins were crosslinked is currently under investigation.

DISCUSSION

In this paper and a previous one (22) evidence is presented that irradiation of polyribosomes results in the bonding of proteins to RNA in such a way that the complexes are stable under conditions that ordinarily disrupt RNA-protein complexes, that is they are stable in the presence of sodium dodecylsulfate and in the presence of high salt and urea. The nature of bonds formed between the RNA and proteins was not investigated, and the possible formation of bonds other than covalent ones has not been formally ruled out. However, according to the extensive literature on the UV photochemistry of proteins and nucleic acids it is almost certain that covalent bonds between the RNA and proteins were formed (see the Introduction for references).

Affinity chromatography on oligo(dT)-cellulose was used to select for RNA-protein complexes containing mRNA, since it is well-established that most mRNA in mammalian cells contains poly(A), and other RNAs present in polyribosomes do not (reviewed in reference 1). It was shown that the binding of protein to the oligo(dT)-cellulose column was UV dose-dependent, and there was little or no effect of irradiation on RNA binding to the column at doses as high as 2×10^5 ergs/mm². Furthermore, the proteins did not rebind to the column if the complexes were treated with RNAases. This finding rules out direct binding of proteins to the column. Also, the bound proteins were eluted in the absence of salt, as expected if the basis for binding was hybrid formation between poly(A) and oligo(dT). Based on these criteria the proteins bound to the oligo(dT)-cellulose column as a result of UV irradiation were most likely crosslinked to poly(A)⁺mRNA.

Six proteins crosslinked to mRNA were identified. They had apparent molecular weights of 98,000, 78,000, 75,000, 68,000, 62,000 and 52,000. Some of these molecular weights, e.g., 78,000, 68,000, and 52,000, are similar to molecular weights of mRNA-associated proteins reported by other investigators who did not use crosslinking (reviewed in references 1,2). For this reason, and also, because the electrophoretic mobilities of the proteins crosslinked to mRNA correspond to the electrophoretic mobilities of major bands present in the labeled proteins of unirradiated polyribosomes, the electrophoretic mobilities of the proteins do not seem to be greatly

altered by crosslinking to RNA, provided that most of the nucleotides are removed prior to electrophoresis.

I found a somewhat larger number of proteins associated with mRNA by the criterion of crosslinking than other investigators found who used chromatography on oligo(dT)-cellulose without crosslinking. For example, Lindberg and Sundquist (4) found proteins of 130,000, 78,000, 68,000, and 56,000 in KB cells, a cultured human cell line. Kumar and Pederson (41), also in human cells (HeLa) found proteins of 120,000, 78,000 and 52,000. Possibly, this difference results from isolating polyribosomes under conditions of low ionic strength so as to minimize dissociation of proteins from RNA in the present work, and also, from stabilizing mRNA-protein complexes by crosslinking prior to carrying out subsequent manipulations. It is not due to irradiating intact polyribosomes, since an identical pattern of protein bands was obtained by irradiating EDTA-released mRNP. It should be noted that proteins of greater than 100,000 daltons were not detected among the proteins crosslinked to mRNA. However, crosslinking of such proteins to mRNA cannot be ruled out, since they would have been obscured by radioactivity that did not enter the gel. It should be noted also that the proteins crosslinked to mRNA in the present work were identified under a particular set of labeling conditions, and that other proteins might be found under other conditions. Also, the possibility that unlabeled proteins were crosslinked to mRNA has not been ruled out.

In agreement with other workers who showed that the poly(A) segment of mRNA is associated with a protein of 78,000 daltons I found that a protein of this size could be crosslinked to poly(A). The evidence was that the protein was rebound to oligo(dT)-cellulose after digesting isolated poly(A)⁺ RNA-protein complexes with RNAases that digest sequences other than poly(A). Furthermore, treatment with a RNAase that hydrolyzes poly(A) was needed to demonstrate the presence of this protein in the gel of the material rebound to oligo(dT)-cellulose. The 78K protein appeared to be the only one crosslinked to poly(A). In this respect my results differ from the results of some workers who found various additional proteins associated with poly(A) (34, 36, 37, 42). The reason for this difference is not entirely clear. However, the others did not use crosslinking, and it is difficult to rule out non-specific binding of proteins to the column and to poly(A) under their conditions. The finding that a protein was crosslinked to poly(A) may be the first report of UV-induced protein-purine crosslinking at the polynucleotide level, although others have reported the UV-induced crosslinking of cyclic

AMP and ATP to proteins (19-21).

These results also show that most of the proteins crosslinked to mRNA, i.e., the 98K, 75K, 68K, 62K, and 52K proteins, were crosslinked to sequences other than poly(A). These proteins were not rebound to oligo(dT)-cellulose after digesting isolated poly(A)⁺mRNA-protein complexes with RNAases that digest sequences other than poly(A). Furthermore, these proteins, in contrast to the protein crosslinked to poly(A), did not require treatment with a RNAase that digests poly(A) in order to enter the gel. Until now there has been little direct evidence for the binding of polyribosomal mRNA-associated proteins to sequences other than poly(A). However, Rosenfeld and Barrieux (43) used an entirely different approach to show that a protein of approximately 68,000 daltons is associated with a sequence other than poly(A). They purified a protein of this size from a 0.5 M KCl extract of rabbit reticulocyte polyribosomes and showed that it was capable of binding to poly(A)⁺mRNA, but not to poly(A), or other RNAs and synthetic polynucleotides. The results of Rosenfeld and Barrieux, as well as my own, suggest that proteins may bind specifically to mRNA sequences other than poly(A), and also, that proteins may be removed from mRNP by washing polyribosomes with 0.5 M KCl, a procedure that is widely used in the isolation of mRNP.

The 78K protein was exceptional in that it was the only one detectable in both the bound and unbound fractions when isolated poly(A)⁺mRNA-protein complexes were digested with RNAases A and T₁ and rechromatographed on oligo(dT)-cellulose. If one assumes that the crosslinking of the 78K protein to mRNA is sequence-specific, then this finding suggests either that there is one 78K protein that can bind to more than one sequence in mRNA, or else that there is more than one protein of this size associated with mRNA. The second possibility is probably the correct one. Setyono (44) analyzed the proteins of conventionally isolated mouse ascites tumor cell mRNP by two dimensional gel electrophoresis and found three proteins of this size which were resolved on the basis of isoelectric point. Only the most acidic of these was associated with poly(A) (B. Setyono, personal communication). We are currently preparing partial peptide maps of the 78K proteins crosslinked to poly(A) and to sequences other than poly(A), and preliminary results suggest that they are indeed distinct proteins (B. Setyono and J. R. Greenberg, unpublished results).

The question arises as to whether or not the UV-induced crosslinking of proteins to mRNA is sequence-specific. A number of studies have shown

that UV-induced crosslinking of proteins to nucleic acids can be extremely sequence-specific in the sense that only a limited number of amino acids are sites for crosslinking to a particular polynucleotide and vice versa. For example, the exact nucleotide of E. coli 16S rRNA to which ribosomal protein S7 is crosslinked, and the oligopeptide of protein S7 to which 16S rRNA is crosslinked have been identified (12, 45). The oligopeptides of RNAase A to which the substrate is crosslinked have been identified (9). Oligonucleotides of specific tRNAs that can be crosslinked to the corresponding aminoacyl tRNA synthetase have been identified (7, 14). The site in the aminoacyl tRNA synthetase that binds ATP has been identified by crosslinking (21). In the present work there are indications that crosslinking of proteins to mRNA by irradiating polyribosomes with UV light is also sequence-specific. Firstly, the set of proteins crosslinked to poly(A)⁺mRNA is clearly a subset of the total labeled polyribosomal proteins. Secondly, only one protein was crosslinked to poly(A), and the other proteins crosslinked to mRNA were crosslinked only to sequences other than poly(A). Clearly, the set of proteins crosslinked to mRNA did not result from UV-induced alterations in ribosome structure, since the same proteins were crosslinked to mRNA whether intact polyribosomes or EDTA-released mRNP were irradiated. These findings seem to warrant further investigation of sequence specificity in the UV-induced crosslinking of proteins to mRNA.

In conclusion, UV-induced crosslinking of proteins to mRNA promises to be a useful tool for investigating mRNA-protein complexes. In the absence of crosslinking it is usually necessary to elute mRNA and proteins from oligo(dT)-cellulose columns with agents that disrupt RNA-protein complexes, and it is not possible to be sure that proteins are bound to the column because of their association with poly(A)⁺mRNA. However, after crosslinking chromatography can be done under conditions that prevent non-covalent binding of proteins to RNA and to the column. Also, after crosslinking it is possible to do repeated cycles of chromatography until a constant level of RNA and protein binding is obtained. There are indications that UV-induced crosslinking of proteins to mRNA is sequence-specific, and the finding that a crosslinked poly(A)-protein complex can bind to oligo(dT)-cellulose suggests that DNA-RNA hybridization may be generally applicable to the investigation of crosslinked RNA-protein complexes.

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