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ULTRAVIOLET LIGHT-INDUCED CROSSLINKING OF TWO MAJOR PHOSPHOPROTEINS AND POLY(A)$^+$RNA FROM FREE POLYRIBOSOMES; CHANGES IN PHOSPHORYLATION BY INHIBITORS OF TRANSCRIPTION AND TRANSLATION

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SUMMARY: Polyribosomes were isolated without the use of detergents, irradiated with ultraviolet light and labelled in the presence of (γ-32P) adenosine 5'-triphosphate. Poly(A)$^+$RNA-protein structures separated by chromatography on oligo(dT)-cellulose contained up to 10 crosslinked proteins as shown by SDS-polyacrylamide gel electrophoresis. These included a 71 kDa poly(A)-bound species and two major phosphoproteins of 66 and 130 kDa. Pretreatment of rats with inhibitors of transcription and translation caused different and significant alterations in the labelling of the two phosphoproteins, suggesting that phosphorylation of proteins closely associated with mRNA may be involved in the regulation of the stability of this RNA or its binding to structural elements in the cell.

In various recent experiments aimed at localizing particular high molecular mass phosphoproteins to nuclear substructures, we have identified complexes of heterogeneous nuclear RNA (hnRNA) and protein that are crosslinked by irradiation with ultraviolet light in both isolated nuclei and whole cells (1). Two major phosphoproteins, of 110-120 and 40-42 kDa, were identified in the crosslinked poly(A)$^+$hnRNA-protein complex; the larger one was shown to be identical with the 110 kDa-phosphoprotein present in purified nuclei and nuclear matrix which we have described in previous communications (2).

In studies on the phosphorylation of ribosomal proteins it was found that significant amounts of the 110 kDa-protein were also present in preparations of free polyribosomes (3). Both this observation and reports by others describing the presence of high molecular mass phosphoproteins in polyribosomes (4-6), prompted us to apply the techniques of ultraviolet irradiation and subsequent analysis of crosslinked proteins to polyribosomes. In this way we hoped to discover whether an mRNA-protein complex is formed, similar to the hnRNA-protein complex and containing phosphorylated proteins in close and direct contact with the RNA. Experiments of this sort should help solve the question of whether the polyribosome-associated fraction of

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the 11o kDa-phosphoprotein is bound to mRNA and involved, for example, in reactions concerning the nucleo-cytoplasmic transfer of this RNA.

MATERIALS AND METHODS

Male Wistar rats weighing 18o-2oo g were starved overnight before isolating liver polyribosomes. Details of treatment of animals with metabolic inhibitors are shown in the text and legends to figures.
In order to label RNA the RNA precursor (6-14C)orotic acid (5o mCi/mmol; Amersham Buchler, Braunschweig, FRG) was injected intraperitoneally into rats 45 min prior to the isolation of ribosomes.
Glassware and some solutions were treated with diethylpyrocarbonate and then autoclaved. Phenylmethylsulfonylfluoride (PMSF) and aprotinin were added as proteinase inhibitors.

Isolation of polyribosomes. Polyribosomes were prepared without the use of detergents following the procedure described in (7) adjusted to work at minus temperature. The homogenization medium was made up with 4o% glycerol and the homogenate cooled to about -15 °C. Prior to high speed centrifugation at 0 °C, 45 ml of the mitochondrial supernatant was mixed with 3o ml 2.o M sucrose. Pellets were frozen in liquid nitrogen and stored at -8o °C.

Irradiation of polyribosomes with ultraviolet light and isolation of the crosslinked mRNA-protein complex. Polyribosomes (8o-1oo OD_{26o} units) were resuspended in 2.5 ml double destilled water plus 1ooo units human placental RNAase inhibitor (Amersham Buchler, Braunschweig, FRG) and diluted to 5 ml with 1oo mM Tris-HCl, pH 7.5, 5o mM KCl, 1o mM $MgCl_2$, 2 mM PMSF, 28 ug/ml aprotinin or 5o mM morpholinopropanesulphonic acid, pH 6.5, 2o mM $MnCl_2$, 2o mM ß-mercaptoethanol, 2o uM Na-vanadate, 2 mM PMSF, 28 ug/ml aprotinin. Following irradiation the ribosomal suspension was incubated in the presence of 5o uCi (γ-32P) adenosine 5'-triphosphate (9-13 Ci/mmol; New England Nuclear, Dreieich, FRG) at 1o °C for 1o min. Treatment of polyribosomes with ultraviolet light and all further steps of isolation of the crosslinked mRNA-protein complex were performed as described in detail previously (1).
In one series of experiments the mRNP bound to the column of oligo(dT)-cellulose was exhaustively washed with 1o mM Tris-HCl, pH 7.4, o.5 M LiCl, 1 mM EDTA, 14 ug/ml aprotinin and 1o ug/ml heat-treated RNAase A at room temperature prior to elution with low salt buffer.

Electrophoretic techniques. Treatment of precipitates with RNAase T_2, SDS-polyacrylamide gel electrophoresis and re-electrophoresis of bands following limited proteolysis by S. aureus protease V 8 were as described previously (3).

RESULTS

Isolation and SDS-polyacrylamide gel electrophoresis of crosslinked proteins.

After irradiation with ultraviolet light polyribosomes were fractionated on oligo(dT)-cellulose into a non-bound portion and the crosslinked poly(A)$^+$RNA-protein complex eluted at low salt concentration. The RNA in the eluate was radioactively labelled when the polyribosomes were from animals treated with (^{14}C)orotic acid; phosphate-labelled proteins were obtained following incubation of polyribosomes with (^{32}P)ATP (diagrams not shown; see ref. 1). Crosslinked proteins in the low salt eluate were analysed by SDS-polyacryl-

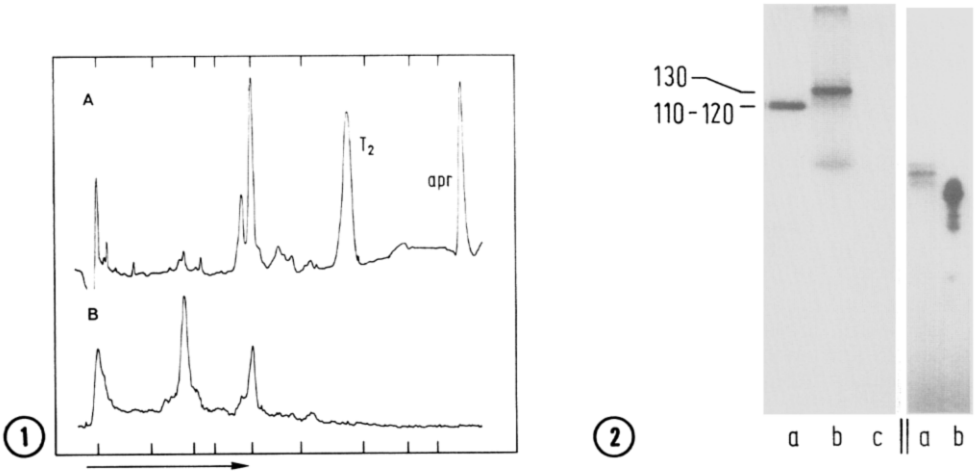

Figure 1. SDS-polyacrylamide gel electrophoresis of proteins cross-linked to polyribosomal poly(A)$^+$ RNA by ultraviolet light. Free polyribosomes were resuspended, irradiated with ultraviolet light and labelled in the presence of (32P) ATP as described in Methods. Poly(A)$^+$ RNA-containing structures were isolated on a column of oligo(dT)-cellulose. RNA was removed by RNAase T_2 prior to electrophoresis. Proteins were localized by staining with Coomassie blue (A) or autoradiography (B). Short vertical lines on the abscissa indicate positions of molecular mass marker proteins as shown in Fig. 3. T_2, position of RNAase T_2; apr, aprotinin.

Figure 2. Left: SDS-polyacrylamide gel electrophoresis of phosphoproteins crosslinked to nuclear and polyribosomal poly(A)$^+$RNA. Nuclear (a) and polyribosomal (b, c) proteins were labelled and isolated as described previously (1) or in the legend to Fig. 1, respectively. Samples in (a) and (b) were irradiated; (c) is a control without irradiation. Right: SDS-re-electrophoresis of phosphoproteins following partial digestion with protease V 8. Major labelled bands in Fig. 2, left, were cut out and analysed as described previously (3). (a), 130 kDa-polyribosomal protein; (b), 110-120 kDa-nuclear protein.

amide gel electrophoresis. They consisted of 2 major components of 66 and 71 kDa, several bands between 42 and 61 kDa, and a characteristic pattern of 5 bands between 108 and 152 kDa as revealed by staining with Coomassie blue (Fig. 1A). The 71 kDa component was presumably bound to the poly(A) segment of mRNA since it was retained on the column after treatment with RNAase A and then eluted as the only protein at low ionic strength (not shown). Two of the bands contained major phosphoproteins, one, of 130 kDa,being labelled to a higher specific activity than the other, of 66 kDa (Fig. 1B).

Control experiments, including rechromatography on oligo(dT)-cellulose of low salt-eluted crosslinked material, fractionation of non-irradiated labelled polyribosomes (Fig. 2 left, c) and fractionation of irradiated polyribosomes previously treated with RNAases A and T_2, showed that there was no unspecific binding of phosphoproteins to the oligo(dT)-cellulose although traces of the 61 kDa species appeared as a contaminant in some cases. Further, no low molecular mass proteins such as ribosomal proteins, histones or

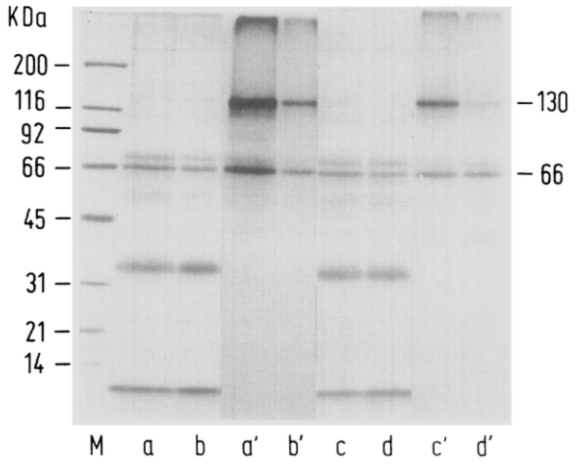

Figure 3. Crosslinked polyribosomal proteins from rats treated with metabolic inhibitors. Proteins were labelled and isolated as described in the legend to Fig. 1. Proteins were from rats following treatment with 2.5 mg/kg cycloheximide (a, a') or 1 mg/kg α-amanitin for 2 h (d, d'), from re-fed animals (b, b') and an untreated control (c, c'). a-d, lanes stained with Coomassie blue; a'-d', corresponding autoradiographs. Positions of 66 and 130 kDa-phosphoproteins are shown. M, molecular mass marker proteins (Bio-Rad Laboratories, München, FRG).

hnRNA-associated core proteins, were observed in the column eluate, apart from small amounts close to the 45 kDa marker protein.

The crosslinked polyribosomal phosphoproteins were compared with nuclear phosphoproteins crosslinked to poly(A)$^+$hnRNA (1). Proteins with molecular masses higher than 100 kDa are predominant in both types of structure (Fig. 2 left). However, further investigation showed that the 130 kDa species from the polyribosome complex and the 110-120 kDa species from the hnRNA complex were not related. They yielded different phosphopeptide patterns when digested by protease V 8 (Fig. 2 right, a and b).

Effect of metabolic inhibitors on the pattern of crosslinked proteins.

In previous crosslinking experiments transcriptional and translational inhibitors were used with nuclei. Similar experiments were performed in order to determine the action of such substances on the crosslinked polyribosomal proteins. Polyribosomes were prepared from control animals and from animals treated with 2.5 mg/kg cycloheximide or 1 mg/kg α-amanitin for 2 h, and submitted to the same procedure of irradiation and fractionation on oligo(dT)-cellulose as described above. The pattern and amounts of stained crosslinked proteins separated by SDS-polyacrylamide gel electrophoresis (Fig. 3, a-d) were very similar to those shown in Fig. 1A irrespective of the pretreatment of animals. The only changes visible were a slight decrease in the intensity of the 130 kDa species and the disappearance of a 48 kDa minor species, after α-amanitin. In contrast large quantitative differences were

observed in the phosphorylation of the crosslinked proteins derived from polyribosomes of pretreated rats (Fig. 3, a'-d'). The translational inhibitor cycloheximide caused a general 2-3 fold increase in phosphorylation of the 13o and 66 kDa major bands as well as all other labelled bands (Fig. 3, a'). The action of α-amanitin was more selective affecting only the labelling of the 13o kDa band which was decreased by about 7o% (Fig. 3, d') as compared to the control. No changes were observed with polyribosomes from rats that were re-fed for 1 h (Fig. 3, b'). The results in Fig. 3 show that the 2 metabolic inhibitors affected the phosphorylation of bands common to all crosslinked samples but did not induce new phosphoproteins or delete proteins already present.

DISCUSSION

The results show that relatively few total proteins are complexed with poly(A)$^+$RNA following ultraviolet irradiation of polyribosomal mRNA-protein structures. This is consistent with previous reports on the composition of proteins crosslinked to mRNA in cultured cells (8, 9). The stained bands with 52 and 71 kDa are considered to belong to the small number of tightly bound mRNP proteins, the latter being the major poly(A)-associated protein (1o, 11). The prominent 66 kDa component, which was not albumin, might be related to proteins of similar size which have been shown to be involved in the binding of polyribosomes to structural elements in the cytoplasm (12).

Crosslinking of the 13o and 66 kDa-phosphoproteins indicated that these species were in direct contact with polyribosomal mRNA. In contrast the 11o kDa-phosphoprotein, which is also observed in preparations of free polyribosomes, appeared to be bound less tightly or in a different way since it was only crosslinked to a small extent in the procedure applied here. The phosphoprotein composition of crosslinked mRNP and crosslinked hnRNP was similar. Both showed two major bands, one large size and one smaller with 13o or 11o-12o kDa, and 66 or 4o-42 kDa, respectively. Phosphoproteins of comparable molecular masses have been described previously in polyribosomal mRNPs and included species of 13o, 52 and 64 kDa or 125, 1o7, 76 and 63 kDa. Most of these were phosphorylated by a casein kinase type II enzyme (5, 6).

The two metabolic inhibitors did not cause more than very small changes in the pattern of crosslinked proteins. These results differ from previous data which suggested that the composition of mRNA-protein particles is significantly altered following inhibition of RNA-polymerase II (13). The reason for the discrepancy is not yet known but it might be explained by differences in the preparation and type of poly(A)$^+$ structures or conditions of the experimental procedure. In contrast the inhibition of transcriptional or translational activities greatly influenced the phosphorylation of

crosslinked proteins. The 13o kDa-band in particular was susceptible to α-amanitin applied in vivo, resembling in this respect the 11o-12o kDa nuclear crosslinked protein (1). After treatment of rats with cycloheximide, phosphorylation of the 13o kDa-band was about 1o times higher than after treatment with α-amanitin. These highly significant changes in phosphorylation suggest that at least one of the two crosslinked factors possesses regulative properties, possibly controlling such parameters as stability of mRNA under different metabolic conditions (14) or its binding to positions in the cytoskeleton (15).

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