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The major RNA in prosomes* of HeLa cells and duck erythroblasts is $tRNA^{Lys,3}$

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ABSTRACT

Two-dimensional gel electrophoresis of HeLa cell prosomal RNAs, 3'-end labeled by RNA ligase, revealed one prominent spot. Determination of a partial sequence at the 3'-end indicated full homology to the 18 nucleotides at the 3'-end of $tRNA^{Lys,3}$ from rabbit, the bovine and the human species. An oligonucleotide complementary to the 3'-end of $tRNA^{Lys,3}$ hybridized on Northern blots with prosomal RNA from both HeLa cells and duck erythroblasts. In two-dimensional PAGE, the major pRNA of HeLa cells co-migrated with bovine $tRNA^{Lys,3}$. Reconstitution of the CCA 3'-end of RNA from both human and duck prosomes, by tRNA-nucleotidyl-transferase, confirmed the tRNA character of this type of RNA. Furthermore, it revealed at least one additional tRNA band about 85 nt long among the prosomal RNA from both species. Finally, confirming an original property of prosomal RNA, we show that *in vitro* synthesized $tRNA^{Lys,3}$ hybridizes stably to duck globin mRNA, and to poly(A)$^+$- and poly(A)$^-$-RNA from HeLa cells.

INTRODUCTION

Prosomes are ubiquitous high molecular weight ribonucleoprotein complexes, composed of a variable set of 28 characteristic proteins with MW ranging from 19 000 to 36 000 and small RNAs of 75 to 120 nucleotides in length (1, 5). Originally, they were discovered as subcomplexes of repressed mRNPs, in part associated with the intermediate filaments of the cytoskeleton (1, 5, 6) and found to inhibit *in vitro* protein synthesis (7). It was later shown that prosomes are identical to a multicatalytic proteinase complex (MCP) first described by Wilk and Orlowski (4, 8, 9). Furthermore, evidence was presented for the participation of this complex in the formation of a 26S proteinase complex, implicated in the ATP-dependent selective breakdown of ubiquitin-conjugated proteins (10, 11) and its involvement in the generation of the MHC class I binding peptides (12, 13).

RNA has been isolated from prosomes purified from organisms as distant in evolution as plants, *drosophila*, duck and the human species and was found to be heterogenous and variable, in function of the cell type (1, 5, 8, 14, 15). However, the presence of this RNA in the complex active in proteolysis had long been a matter of controversy. Using a variety of purification procedures, the groups of Rivett, Kloetzel and our own clearly demonstrated the presence of prosomal RNA (pRNA) in the proteolytically active complex (8, 16, 17); furthermore, the dissociation of the complex into its subunits was shown to be a prerequisite for nuclease digestion of the pRNA (17).

No precise function can as yet be ascribed to the prosome particle or its RNA, although some data suggest an involvement in mRNA function and/or protein metabolism. This is indicated by the capacity of isolated prosomal RNA to hybridize to mRNA and to inhibit *in vitro* protein synthesis, as shown for the intact prosome particle. However, for further functional analysis of this prosomal constituent, the RNA sequence was of importance. Therefore sequencing of the RNA of highly purified prosome particles from HeLa cells was initiated.

MATERIAL AND METHODS

Materials

[^{32}P]pCp (3000 Ci/mmol), [α^{32}P]ATP (3000 Ci/mmol, 10 mCi/ml) and [γ^{32}P]ATP (5000 Ci/mmol) were obtained from Amersham (England).

Cell culture

HeLa cells were grown in suspension at 4×10^5 cells/ml with a generation time of about 24 h in Eagle's Minimum Essential Medium supplemented with 10% newborn calf serum.

*The term 'Prosome' introduced by our laboratory (1) for the then unknown particle is used here and, speaking of its protease activity, the term 'Multicatalytic Proteinase or MCP' following the recommendation of the group of the enzymologists concerned (Dahlman *et al.* (2), Orlowski and Wilk (3)), in preference to the term 'Proteasome' suggested by Arrigo *et al.* (4).

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Cell fractionation and purification of prosomes from HeLa cells and duck erythroblasts

The isolation and purification of prosomes from either HeLa cells or duck erythoblasts have been reported (5). The protein concentration of the purified particle preparations was determined by the method of Bradford (Biorad) using BSA as the standard.

Dissociation of prosomes by Zn^{2+}

Prosomes at a concentration of 0.5 to 1 $\mu g/\mu l$ were incubated at 37°C in the presence of 1 mM Zn^{2+}. After 1 h, 1 mM EDTA was added prior to nuclease digestion.

Nuclease protection assay and labeling of RNA with RNA ligase

The samples (10−100 μg prosomes) were digested in a final volume of 50 μl by ribonuclease V1 (25 U/ml), in a buffer containing 20 mM Triethanolamin (pH 7.4), 200 mM NaCl and 10 mM $MgCl_2$. The reaction was stopped after 20 min at 37°C by adding 200 μl of phenol−chloroform. After extraction, the RNA was labeled at the 3′-end with [^{32}P] pCp in a reaction catalyzed by T4 RNA ligase. The RNA fragments were analyzed by electrophoresis in 10% acrylamide/8M urea gels followed by autoradiography.

Labeling of pRNA with tRNA-nucleotidyltransferase

Prior to the labeling reaction, the 3′-end (i.e. CCA) was removed by digestion with phosphodiesterase. The RNA of purified prosomes was incubated for 10 min at 20°C in 10 μl buffer, containing 0.5 μg phosphodiesterase, 50 mM Tris−HCl (pH 8.0) and 10 mM $MgCl_2$. The reaction was stopped by adding phenol-chloroform, followed by two additional chloroform extractions. The reaction mixture for labeling of the truncated RNA by tRNA-nucleotidyltransferase (from *E. coli*) contained in 10 μl the RNA, 50 mM Tris−glycine (pH 8.9), 8 mM dithiothreitol, 7 mM $MgCl_2$, 6 μg tRNA-nucleotidyltransferase, 3.3 pmoles [α^{32}P]ATP and 50 μM CTP. After incubation for 1 h at 37°C, the RNA was phenol−chloroform extracted prior to analysis by gel electrophoresis.

Labeling of deoxyoligonucleotides with [γ^{32}P]ATP by polynucleotide kinase

The labeling mixture (20 μl) contained 10 pmoles deoxy-oligonucleotides, 50 mM Tris−HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, 1 mM spermidine, 15 pmol [γ^{32}P]ATP and 7.5 U polynucleotide kinase. After incubation for 30 min at 37°C and subsequently 10 min at 68°C, the labeled oligonucleotides were purified on NAP 10 columns (Pharmacia) in 4×SSC.

Purification of radioactively labeled prosomal RNA on one- or two-dimensional polyacrylamide gels

One-dimensional polyacrylamide gel analysis of the RNA was carried out by electrophoresis in 10% acrylamide/8 M urea gels, using TBE buffer (0.089 M Tris−borate (pH 8.3), 0.002 M EDTA). The run was continued until the dye (bromophenol blue) reached the bottom of the gel.

The first dimension of two-dimensional gel electrophoresis was either on 12% PAGE in 6 M urea (pH 3.5) or on 10% PAGE in 4 M urea. In the former case, for 50 ml, 12.5 ml acrylamide stock solution (40% acrylamide, 1.36% N,N′-methylene bis-acrylamide), 18 g urea and 2.5 ml of 1 M citric acid were mixed prior to initiation of polymerization by adding 0.2 ml $FeSO_4$ (2.5 mg/ml), 0.2 ml ascorbic acid (100 mg/ml) and 40 μl H_2O_2.

Electrophoresis was carried out at 4°C and 800 V overnight in a buffer containing 4 mM EDTA and 25 mM sodium citrate (pH 3.5), until the dye (xylene cyanol) reached the bottom of the gel. In the case of 10% PAGE in 4 M urea as conditions for the first dimension, a stacking gel was used in addition. For 35 ml, 5 ml 'acrylamide stock solution' (38% acrylamide and 2% N,N′-methylene bis-acrylamide) were mixed with 8.4 g urea, 0.5 ml 150 mM Tris−HCl (pH 6.7) and water up to 35 ml. The run was for 48 h at 300 volts and 4°C. The second dimension was by 20% PAGE in 8 M urea in TBE buffer, or by 12% PAGE and 8 M urea in TBE buffer when the first dimension was acidic. To elute the RNA, the corresponding gel slices were immersed and soaked for several hours in 0.5 M ammonium acetate, 0.01 M magnesium carbonate, 0.1% SDS, 0.1 mM EDTA, (pH 5).

Sequence determination of randomly hydrolyzed RNA by two-dimensional polyacrylamide gel electrophoresis

Prosomal RNA was hydrolyzed for 2 h or 4 h at 95°C in H_2O in the presence of 1 $\mu g/\mu l$ of yeast tRNA as a carrier. Electrophoresis in the first dimension was on an acidic gel as described above, but migration was at 700 V for 4−5 h.

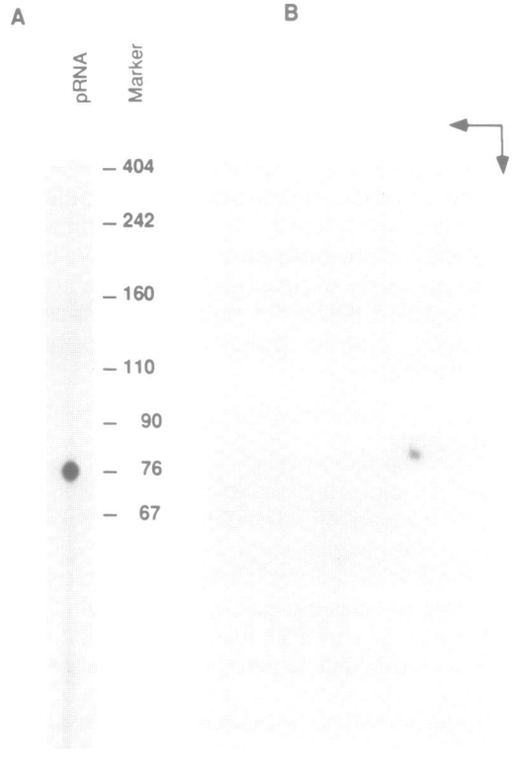

Figure 1. One- and two-dimensional gel electrophoresis of [^{32}P]pCp-labeled prosomal RNA. Purified prosomes of HeLa cells were phenol-extracted and, prior to PAGE analysis, the RNA was labeled with [^{32}P]pCp and RNA ligase. (A) One-dimensional electrophoresis in a 10% polyacrylamide−8 M urea gel in TBE. (B) Two-dimensional gel electrophoresis of [^{32}P]pCp labeled prosomal RNA. Migration in the first direction (10% PAGE in 4 M urea) is from right to left; migration in the second direction (12% PAGE in 8 M urea) is from top to bottom. The spot was eluted, purified and sequenced.

Electrophoresis in the second dimension was performed in an inclusion gel of 18% polyacrylamide/7 M urea and a running gel of 23% polyacrylamide−7 M urea, both in TBE buffer for 48 h at 300 Volts.

Primary structure determination of randomly hydrolyzed RNA by electrophoresis and homochromatography

High-voltage electrophoresis−homochromatography was carried out as described by Keith (18), using cellulose acetate electrophoresis in the first, and DEAE−cellulose homochromatography in the second dimension.

Northern blot hybridization

The pRNA was fractionated by electrophoresis in a 10% polyacrylamide/8 M urea gel in TBE. After transfer by capillarity onto a 'GeneScreen Plus' hybridization transfer membrane (Du Pont) in 10×SSC, the membrane was baked at 80°C for 2 h. After prehybridization for 3 h at 55°C in 4×SSC, 0.01% (w/v) Ficoll, 0.01% (w/v) polyvinylpyrrolidone, 0.01% (w/v) bovine serum albumin, 0.1% sodium pyrophosphate, 1% SDS and 150 μg/ml denatured salmon sperm DNA, labeled oligodeoxynucleotides were added. Hybridization was overnight at 55°C. Subsequently, the blot was rinsed twice in 2×SSC and 0.5% SDS at room temperature, and then washed twice in 2×SSC and 0.5% SDS at room temperature for 10 min under constant stirring, and thereafter once in 2×SSC and 0.5% SDS at 55°C for 12 min under constant stirring. The membrane was exposed to Fuji X-ray films.

Dot-blot hybridization

RNA samples (0.2 or 1 μg) were spotted onto 'GeneScreen Plus' hybridization transfer membrane according to the manufacturer's protocol and baked at 80°C for 2 h. After prehybridization at 42°C, hybridization was carried out for 16 h with 9×10^5 cpm/ml [^{32}P]-labeled RNA. The prehybridization and hybridization mixtures contained 50% formamide, 0.01% (w/v) Ficoll, 0.01% (w/v) polyvinylpyrrolidone, 0.01% (w/v) bovine serum albumin, 5×SSC, 0.1% sodium pyrophosphate, 1% SDS and 150 μg/ml of denatured salmon sperm DNA. After hybridization the filter was washed several times at increasing stringency and exposed to a Fuji X-ray film. In the final conditions the filter was washed twice in 2×SSC at room temperature for 5 min; twice in 1×SSC and 1% SDS at 65°C for 10 min; twice in1×SSC and 1% SDS at room temperature for 30 min prior to be exposed to a Fuji X-ray film.

RESULTS

To characterize the prosomal RNA from HeLa cells, purified prosomes were exposed to nuclease digestion by the enzyme V1, specific for double stranded RNA, prior to phenol-extraction. The RNA content was then 3′-end labeled with [^{32}P]pCp and analyzed by one-dimensional gel electrophoresis. The result (Figure 1A) revealed a major RNA of about 75 nucleotides (nt) in length and occasionally an additional faint RNA band approximately 110 nt long. It seemed, however, that the RNA in the 75 nt range migrated as a smear rather than as a sharp band, apparently confirming previous results which indicated a heterogenous RNA population in prosomes from HeLa cells (1, 5). To elucidate this point, two-dimensional gel-electrophoresis of prosomal RNA from HeLa cells was carried out. In the first dimension (12% polyacrylamide gel at pH 3.5), separation was based on the charge of each individual RNA. Thus, it depended on the base composition rather than on chain length, thereby allowing the separation of RNA molecules within the same size range. The second dimension was by 12% PAGE at pH 8.3. As shown in Figure 1B, again one prominent spot was observed with some minor spots of smaller size. In order to obtain some

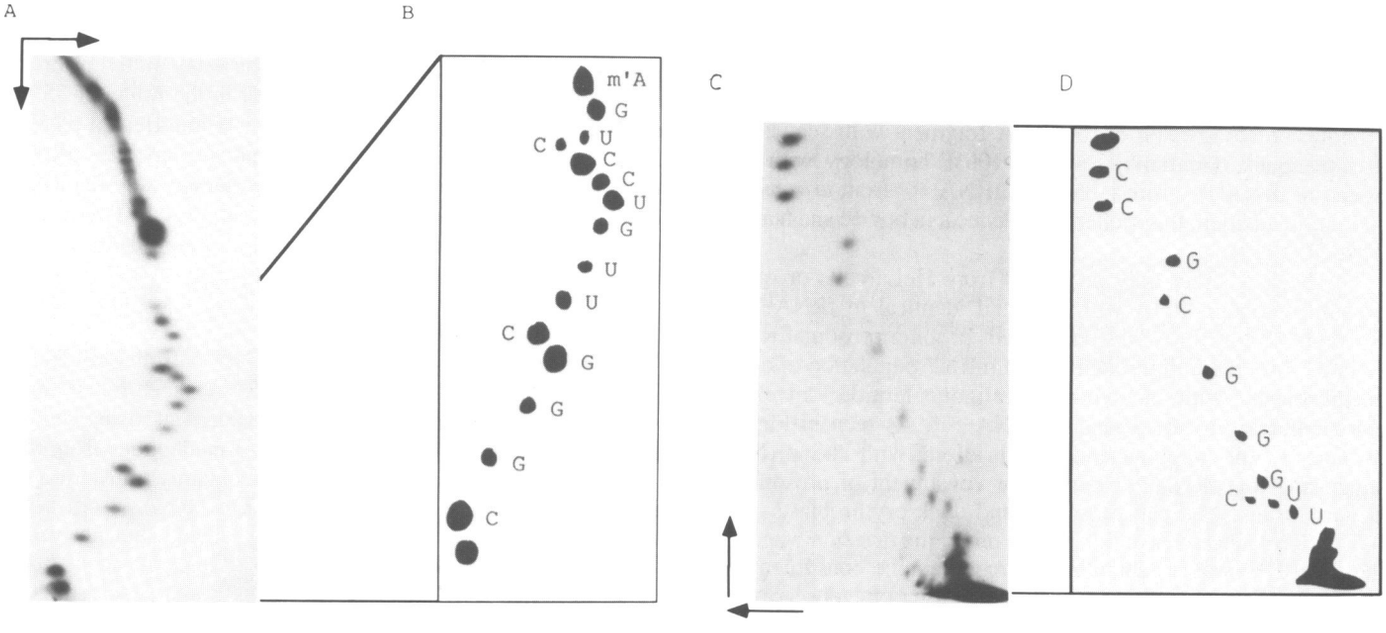

Figure 2. Two-dimensional gel electrophoresis or electrophoresis−homochromatography of randomly hydrolyzed [^{32}P]pCp-labeled prosomal RNA fragments. After bi-dimensional PAGE (Figure 1) and elution of the 3′-end labeled pRNA, the RNA was randomly hydrolyzed and the fragments analyzed by two-dimensional gel electrophoresis or by electrophoresis−homochromatography. (**A**) Autoradiography of two-dimensional gel electrophoresis. (**B**) Its schematic representation and the corresponding sequence. (**C**) Autoradiography of electrophoresis−homochromatography. (**D**) Its schematic representation and the corresponding sequence.

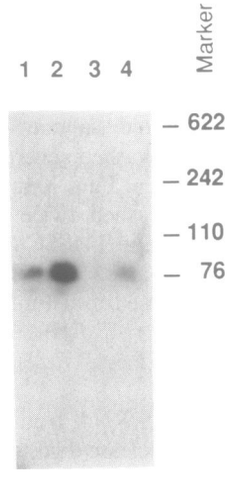

Figure 3. Northern blot hybridization of pRNA to oligonucleotides complementary to the 3′-end of rabbit $tRNA^{Lys,3}$. Hybridization assays were carried out with labeled DNA-oligodeoxynucleotides complementary to the 18 nt at the 3′-end of $tRNA^{Lys,3}$ (5′-TGGCGCCCGAACAGGGAC-3′). Hybridization was overnight at 55°C. Subsequently, the membrane was washed and then exposed to a Fuji X-ray film. Lane (1) pRNA corresponding to 25 μg of prosomes purified from 20S free globin mRNP of duck erythroblasts; lane (2) pRNA corresponding to 50 μg HeLa cell prosomes; lane (3) pRNA corresponding to 25 μg HeLa cell prosomes, dissociated by Zn^{2+} prior to digestion by nuclease V1; lane (4) pRNA corresponding to 25 μg HeLa cell prosomes exposed to nuclease V1 digestion prior to phenol-extraction.

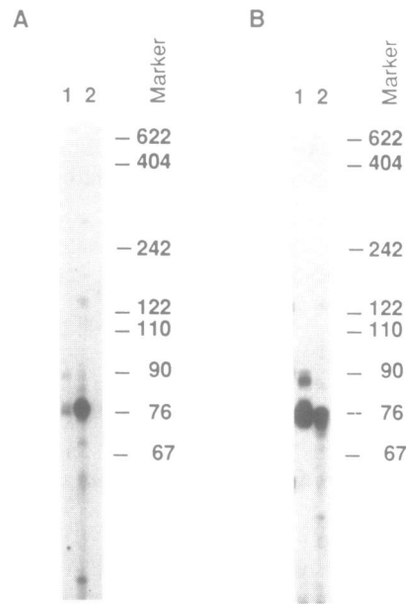

Figure 4. One-dimensional gel electrophoresis of pRNA labeled by RNA ligase or by tRNA-nucleotidyltransferase. After phenol-chloroform extraction, 10 μg of prosomes purified from either (**A**) HeLa cells or (**B**) duck erythroblasts were phenol−chloroform extracted and labeled either by a reaction catalyzed by tRNA-nucleotidyltransferase (lane 1) or by T4 RNA ligase (lane 2) as described in 'Materials and Methods'.

sequence information of the major RNA, the 3′-end labeled RNA was randomly hydrolyzed after elution from the second dimension and the fragments were analyzed by two-dimensional PAGE. This technique allowed to sequence 15 nucleotides close to the 3′-end of the RNA: 5′-GUCCCUGUUCGGGC-3′ (Figure 2A,B). The 3′-end still lacking was completed by alkaline hydrolysis and by electrophoresis−homochromatography, which revealed the sequence 5′-GCCA-3′ for the 3′-end of the RNA (Figure 2C,D). All these techniques together gave a sequence of 18 nucleotides starting from the 3′-end of the RNA, which is: 5′-GUCCCUGUUCGGGCGCCA-3′.

Sequence comparison of this RNA fragment with sequences in a databank (GenBank) revealed 100% homology with the 3′-end of $tRNA^{Lys,3}$ from rabbit, and $tRNA^{Lys,5}$ from *drosophila* and rat. In addition, this sequence is identical in bovine and human $tRNA^{Lys,3}$ (19).

To check whether only prosomes from HeLa cells or other prosomes contain RNA with a 3′-end identical to $tRNA^{Lys,3}$, Northern blot analysis was performed, probing prosomal RNA isolated from the untranslated globin mRNP population of duck erythroblasts with a synthetic oligonucleotide, inverse-complementary to the 3′-end of $tRNA^{Lys,3}$. As seen in Figure 3, lane 1, the oligonucleotide hybridized with the pRNA, suggesting that $tRNA^{Lys,3}$ is a major constituent of prosomes, at least from HeLa cell prosomes and duck erythroblasts.

The tRNA character of this pRNA raised the question whether the $tRNA^{Lys,3}$ was a contamination or a genuine constituent of prosomes. To answer this issue, we made use of the observation that prosomes can be dissociated selectively into their subunits by 10^{-4}M Cu^{2+} or Zn^{2+} and that such disruption of the complex is a prerequisite for nuclease digestion of the RNA content (17). This was indicated by the fact that after Zn^{2+} treatment and subsequent digestion by nuclease V1, an enzyme which cleaves non-specifically double stranded RNA, the enzyme RNA ligase failed to label any RNA fragment in the range of 80 nucleotides. Consequently, prosomes of HeLa cells, Zn^{2+} dissociated or not, were exposed to nuclease digestion prior to Northern blot analysis of their RNA content, using the synthetic oligonucleotide as the probe. As seen in Figure 3, lane 3, dissociation of the complex by Zn^{2+} prior to nuclease digestion abolished all hybridization signal, even of RNA molecules in a lower size range. When Zn^{2+} dissociation was omitted prior to nuclease digestion, a hybridization signal in the range of 75 nt was observed, identical to the size range of non-treated pRNA (Figure 3, lanes 2 and 4). Note that in lane 2, pRNA corresponding to 50 μg prosomes and in lane 3 and 4, pRNA corresponding to 25 μg prosomes were analyzed. This result strongly indicates that this prosomal RNA, likely to be $tRNA^{Lys,3}$, is almost completely, if not totally, protected by the prosomal protein complex against nuclease digestion and is, hence, an intrinsic part of prosomes.

To confirm the tRNA character of this RNA from both HeLa cells and duck erythroblasts, a tRNA specific 3′-end removal/reconstitution experiment was carried out, using venom phosphodiesterase to remove up to 2 or 3 nucleotides from the 3′-end of the tRNAs, and tRNA−polynucleotidyltransferase to reconstitute specifically the terminal CCA. RNAs from both HeLa cells and duck erythroblasts were labeled, but not yeast 5S RNA (Figure 4 A,B, lane 1). The tRNA pattern of both species revealed a tRNA of 75 nt in length and, interestingly, an additional RNA of approximately 85 nt when compared to the pattern of RNA ligase labeling of the same RNA (Figure 4 A,B lane 2). It seems thus that some of the 3′-ends of prosomal RNAs are either transiently blocked by an as yet unknown mechanism or that structural conformations might occasionally

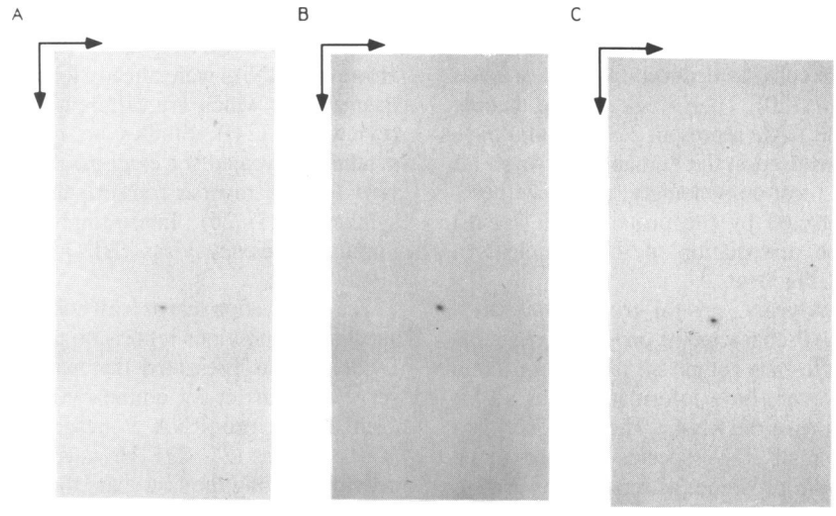

Figure 5. Comigration of pRNA with $tRNA^{Lys,3}$ in two-dimensional gel electrophoresis. [^{32}P]pCp-labeled pRNA of HeLa cells and human $tRNA^{Lys,3}$ were purified by two-dimensional gel electrophoresis, and $tRNA^{Lys,3}$ and the major pRNA thought to correspond to this tRNA were eluted. 1500 cpm pRNA and 4500 cpm $tRNA^{Lys,3}$ were either analyzed separately or mixed together prior to two-dimensional PAGE, migration in first dimension (10% PAGE in 4 M urea) was from left to right and in second dimension (20% PAGE in 8 M urea) from top to bottom. (**A**) 1500 cpm pRNA. (**B**) 4500 cpm human $tRNA^{Lys,3}$. (C) Mixture of 1500 cpm pRNA and 4500 cpm human $tRNA^{Lys,3}$.

impair labeling by RNA ligase. This might be one explanation for the variation in the pRNA, shown in different publications (1, 5, 8, 14, 15). The results obtained by labeling furthermore indicate that $tRNA^{Lys,3}$ is not the only tRNA among the genuine RNAs of prosomes, but that among them another tRNA molecule, about 85 to 90 nt in length, is also present. The latter tRNA may correspond either to Leu, Ser or Tyr specific tRNAs; those are the only ones known as yet, which carry long extra arms, compatible with a length of about 85 nt.

To address the question whether the 75 nt long prosomal RNA in HeLa cells is indeed $tRNA^{Lys,3}$, the major pRNA was compared to genuine $tRNA^{Lys,3}$ by mixing them prior to two-dimensional gel electrophoresis (Figure 5). Both tRNAs co-migrated on the gel, indicating their identity. Same behaviour upon gel electrophoresis and same 3'-end sequence show — since $tRNA^{Lys,3}$ are identical in rabbit, bovine and human — that the pRNA found in HeLa prosomes may correspond to human $tRNA^{Lys,3}$.

The ability to hybridize in quite stringent conditions to mRNA was put forward in the past as a characteristic property of pRNA (5, 7). Therefore, to check whether the $tRNA^{Lys,3}$ has the same capacity, dot-blot hybridization experiments were carried out with a [^{32}P]-labeled *in vitro* transcript of a bovine $tRNA^{Lys,3}$ construct. It was found, that, indeed, this tRNA transcript hybridizes stably to duck globin mRNA, and to poly(A)$^+$ mRNA and poly(A)$^-$ RNA from HeLa cells, even after washing in stringent conditions (1×SSC, 65°C). Other polynucleotides such as poly(A) or tRNAs from either *Echerichia coli* or calf liver gave no and duck ribosomal RNA only a very weak hybridization signal, when probed with the $tRNA^{Lys,3}$ transcript (Figure 6). Similar results were obtained, when total bovine tRNA was used as a probe instead of $tRNA^{Lys,3}$; only the hybridization with globin mRNA was weaker (data not shown). The strong signal obtained for the globin mRNA when probed with $tRNA^{Lys,3}$ was supported by sequence comparison. This analysis revealed considerable extent of complementary sequences between various avian globin mRNAs and $tRNA^{Lys,3}$.

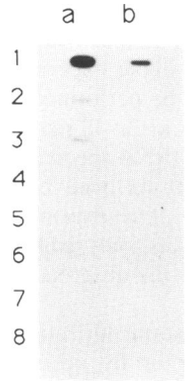

Figure 6. Dot-blot assay of the hybridization of an *in vitro* bovine $tRNA^{Lys,3}$ transcript with various RNAs. RNA or oligonucleotide samples (panel (a) 1 μg; panel (b) 0.2 μg) were spotted onto a 'GeneScreen Plus' hybridization membrane and incubated with [^{32}P]-labeled bovine $tRNA^{Lys,3}$ transcripts as described in 'Material and Methods'. (1) duck globin mRNA; (2) poly(A)$^+$-mRNA from HeLa cells; (3) poly(A)$^-$-RNA from HeLa cells; (4) *Echerichia coli* tRNA; (5) calf liver tRNA; (6) duck ribosomal RNA; (7) poly(A); (8) H_2O.

DISCUSSION

The results, presented here, demonstrate that at least two tRNAs, one of them $tRNA^{Lys,3}$, are part of the prosome particle in HeLa cells and duck erythroblasts. Although we can not fully exclude a contamination of prosomes by these molecules, several lines of evidence presented here and elsewhere, argue strongly against this hypothesis. (i) Several quite distinct purification procedures always led to the recovery of pRNAs in the range of 75−120 nt after phenol extraction of prosomes and 3'-end labeling of the RNAs in the aqueous phase (5, 16). (ii) Only one prominent and specific RNA band, $tRNA^{Lys,3}$ in the present case, was observed in five different preparations, rather than the whole spectrum of tRNA molecules, as expected in the case of a contamination. (iii)

Prosomes do not bind tRNAs non-specifically (data not shown). (iv) The $tRNA^{Lys,3}$, recognizing the codon AAA, is likely to be the minor $tRNA^{Lys}$ in human cells, as deduced from the analysis of codon usage by Aota *et al.* (20). Their work showed that the two Lysine codons AAA and AAG represent 2.38% and 3.54% respectively of all the codons used in the human mRNAs so far sequenced. (v) Finally and most convincingly, as shown here, the prosomal RNA is protected by the protein shell against nuclease digestion, and the dissociation of the particle is a prerequisite for subsequent digestion.

Indeed, within the last few years, several groups established quite unambiguously the RNP-character of prosomes, i.e. that at least some of these particles are composed of both a protein and an RNA moiety. However, little information was so far available concerning the nature of the RNAs. The only exception were some sequences of small RNA species claimed to be associated with the *Drosophila* prosome, as reported by Arrigo *et al.* (15). Among these data, the partial sequence analysis of an RNA of 110 nt revealed that this RNA contained a high proportion of U residues (45%) and that it was clearly different from 5S RNA. The sequence of another prosome associated RNA in the 4S range, revealed a primary structure clearly distinct from tRNAs but 74% homologous to mammalian U6 small nuclear RNA, whereas two other RNAs in the same size range had no homology to any known RNA.

An RNA of about 110 nt in length was occasionally also seen in our preparations of prosomes from HeLa cells. Due to very low radioactive labeling in our experiments, a sequence analysis of this RNA could not be performed.

The analysis of pRNA in the 4S range from HeLa cells, where we detected at least two tRNA species, one being $tRNA^{Lys,3}$, did not confirm the sequence data found for the RNAs in the 4S range examined by Arrigo (15). One reason for this discrepancy might be that the prosomal RNAs analyzed belong to different species. Another cause might be the different labeling methods used or a contamination.

Prelimilary attempts at quantification of the prosomal RNAs by radioactive labeling or by optical density measurements indicate that less than one molecule of RNA is present per particle. This might be due to the fact that our analyses were performed on highly purified prosomes which were even exposed in a final purification step to nuclease digestion prior to phenol-extraction. This criteria, i.e. nuclease digestion for genuine prosomal RNA might also account for the rather simple prosomal RNA pattern presented here, when compared to previous, more complex ones. On the other hand, the non-stoichiometric relationship of RNA to protein in prosomes may suggest that the pRNA participates in only one or a few of the processes within the broad range of putative function(s) in mRNA and protein metabolism that have been claimed to be associated with prosomes (1, 5, 10, 11, 21–23). The original suggestion made by Schmid *et al.* (1), that prosomal RNA may interact with and somehow mediate selective mRNA repression was later substantiated by the demonstration of stable hybridization of prosomal RNA to duck globin mRNA and poly(A)$^+$-mRNA from HeLa cells (5, 7). Furthermore, Horsch *et al.* showed that the hybridization of pRNA is even stronger with poly(A)$^+$-mRNA from adenovirus-infected HeLa cells and that these mRNAs were selectively inhibited in *in vitro* protein synthesis by both prosomes and pRNA. The data indicated the capacity of pRNA to recognize mRNA and, furthermore, to discriminate between mRNA of adenovirus-infected and non infected cells.

Our present work which indicates that at least some of the pRNAs are tRNAs might surprise conceptually at a first glance. However, tRNAs were already found to take part in many cellular mechanisms which are different from protein synthesis (for a review see (24)). Studies on retroviral RNA-directed DNA synthesis revealed for example, that tRNAs act as a primer *in vivo* for the reverse transcriptase in the initiation of cDNA synthesis (25, 26). Interestingly, in the case of the human immunodeficiency virus (HIV), this RNA is $tRNA^{Lys,3}$ also (19).

The observation that at least some pRNAs are tRNAs may also substantiate previous reports on possible functions of prosomes. Evidence was presented that particles, resembling prosomes, serve as a carrier for aminoacyl transferase I or copurify with a eukaryotic pre-tRNA 5′ processing nuclease from *Xenopus laevis* oocytes (21, 23). However, no definitive evidence was provided during the last years that either one of these activities could be an intrinsic enzymatic property of prosomes. The observation of Castano was challenged by recent work of Doria *et al.* who reported the purification of a 5′-processing nuclease from *Xenopus laevis* oocytes in conditions avoiding the contamination by the large cytoplasmic cylindrical particle (27). Speculation in these directions seems therefore unsubstantiated.

However, interesting data emerge from recent work on the involvement of tRNAs in the regulation of the ubiquitin-mediated proteolytic pathway. The MCP activities of prosomes are claimed to play an important role in this degradation process via their participation in a 26S proteolytic complex, responsible for the breakdown of ubiquitinylated proteins. Data by the group of Ciechanover demonstrated the need of some tRNA for the post-translational conjugation of a basic amino acid to proteins with acidic amino terminus. This modification is required for their ubiquitinylation and subsequent degradation (28). If the prosomal tRNAs, especially $tRNA^{Lys}$, were charged with amino acids, an involvement of prosomal RNAs in such a process might be considered. This hypothesis, however, is hampered by several, at a first glance, contradictory results. The data demonstrating the protection of pRNA against nuclease digestion on one hand, and the reported RNAse sensitivity of the conjugation of basic amino acids on the other hand seem contradictory (29). Furthermore, the capacity to label the prosomal $tRNA^{Lys,3}$ at its 3′-end seems to indicate that at least a part of it is not aminoacylated, since lysine would be attached to the same 3′-side OH-group of the ribose (30). However, we cannot exclude that the prosomal $tRNA^{Lys,3}$ could be deaminoacylated during the purification and extraction procedures or that dynamic equilibria might exist *in vivo* between alkylated and free 3′-ends, allowing to reconcile apparent contradictions.

Furthermore, the amount of tRNAs, bound to prosomes might be in correlation with the free tRNA pool in the cell and hence trigger or modulate ubiquitinylation and/or subsequent degradation, or modify via the prosomes the effiency of mRNA translation and thus regulate protein synthesis. Thus, one role of the prosome-MCP particle might be similar to that one proposed for the glucocorticoid receptor by Ali and Vedeckis (31), based on its tRNA binding capacity, in particlular for those coding for the three basic amino acids arginine, lysine and histidine.

To further elucidate this hypothesis as well as those discussed above, a lot of additional data will be necessary, and in particular to know: (i) how many other RNAs and tRNAs are part of the prosomal RNAs; (ii) whether or not the tRNA(s) found in

prosomes are species specific; (iii) whether or not the prosomal tRNAs are aminoacylated *in vivo*, and most important (iv) what fraction of the prosomes integrate an RNA at a given time and whether there is a dynamic equilibrium in between the protein complex and RNP forms of prosomes. Work is in progress to address some of these questions.

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